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CANCER RESEARCH

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Progressive Growth Stages in the Development of Spontaneous Thyroid Tumors in Inbred Swordtails *Xiphophorus montezumae**

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Almost 50 years ago Gaylord and Marsh (5) and Marine and Lenhart (11, 12) showed that thyroid tumors in hatchery-reared trout developed from hypertrophied and hyperplastic thyroid tissue. The tumors invaded and replaced muscle and bone; one tumor metastasized to the wall of the rectum. Gaylord and Marsh classified the tumor as a carcinoma, but Marine and Lenhart regarded it as a severe endemic goiter, because (*a*) iodine inhibited the hyperplasia, (*b*) the incidence of the tumor was directly related to the quality of the water, and (*c*) the tumor appeared in young fish and often regressed with age. Schlumberger and Lucké (15) suggested that all the thyroid tumors in trout might not be alike; most of them could be extreme hyperplastic growths, while the one that metastasized was a true carcinoma.

The present report will describe the developmental stages of the thyroid tumor in inbred laboratory-reared Montezuma swordtails. The thyroid tissue of these fishes will be compared to similar material obtained from wild swordtails taken from their natural habitat and fixed at the site of their capture, in Mexico.

MATERIALS AND METHODS

The laboratory-reared swordtails have been propagated since their initial introduction in 1939 by methods described by Gorbman and Gordon (8) and Gordon (9). The fish have been kept in well conditioned fresh water that, for many years, has been used over and over again. The conditioned water has been interchanged among the tanks containing members of six other species of xiphophorin fishes. The diet of all fishes contained dried, shredded ocean shrimp, liver, cereal, and living tubificid worms; in addition, the young fish were fed live daphnids.

The present report is concerned primarily with an analysis of the thyroidal tissue in two laboratory strains of swordtails (Figs. 1 and 2 and Chart 1). The fish of strain 38[3] represent seven generations of inbreeding, the last three being brother to sister matings. The members of strain 43 have a similar genetic history for the first four generations; the last three generations represent close inbreeding but not by brother to sister matings.

For a histological study of the thyroid follicles, the lower jaw of each fish was removed and fixed in Bouin's fluid. The jaws of the older fish required decalcification with nitric acid and phloroglucin. The tissues were sectioned at 6 μ and stained with either Mayer's hematoxylin-eosin or Masson's trichrome stain.

The height of the epithelial cells of the thyroid was measured by a modification of the method suggested by Rawson and Starr (14), who measured the greatest height of one epithelial cell from each of 200 different follicles. Because most Montezumas did not have 200 follicles, we measured either one cell from each follicle or one cell from each of 50 different follicles. To avoid measuring the same cell twice, 24 sections were skipped between critical sections.

Since the epithelial cells in each follicle varied greatly in size in fishes with hypertrophied tissue, the proper selection of a particular cell for measurement was difficult. Preliminary test analyses were made, first by measuring the smallest, then an intermediate, and finally the tallest thyroid follicle cells in each of several fish. In each series the mean height of epithelial

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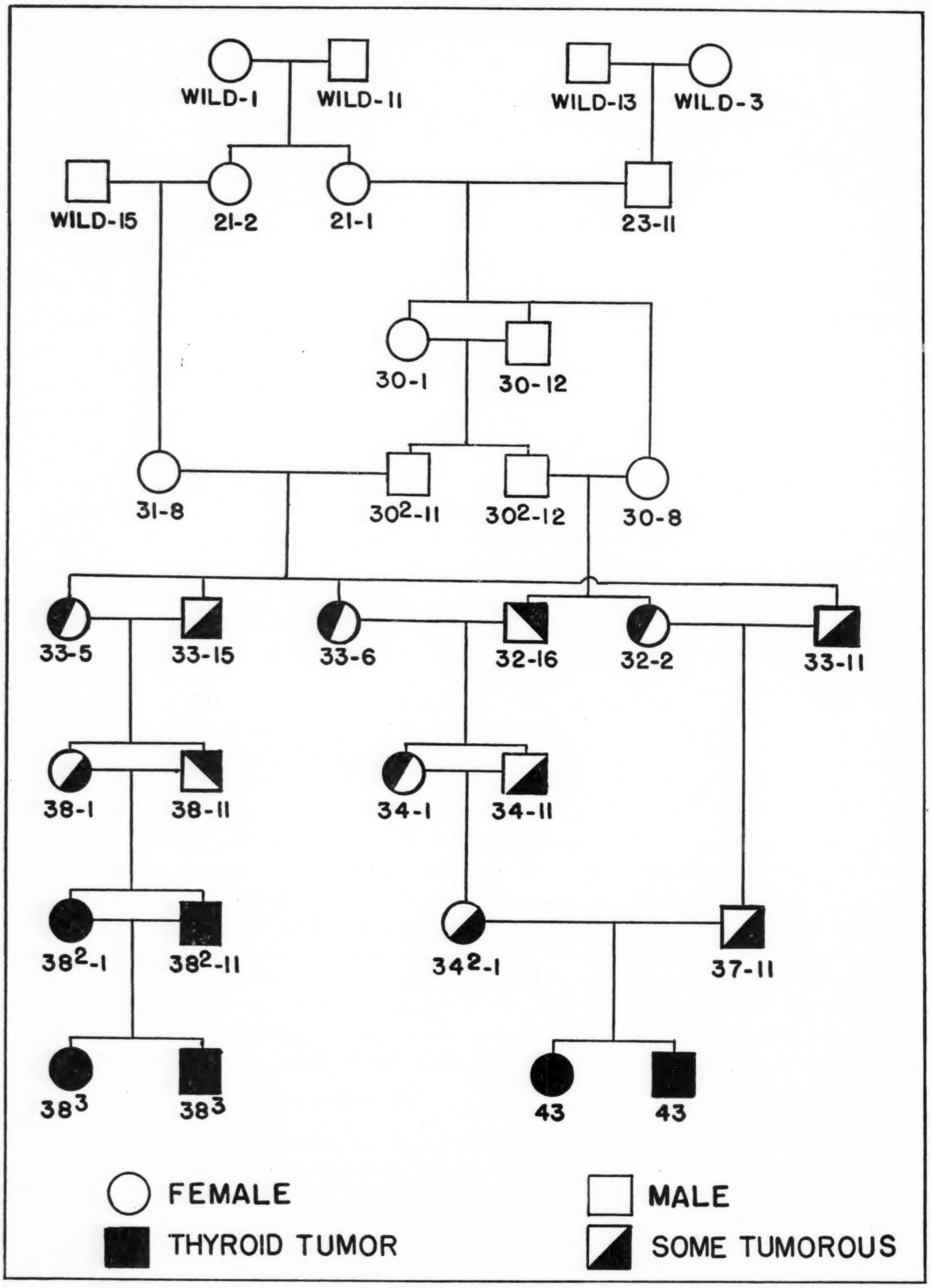

CHART 1.—The genetic history of two laboratory strains of Montezuma swordtails, 38^3 and 43. They are the seventh-generation descendants of those collected in 1939 from Mexico. All the members of the seventh generation developed tumors, although tumors appeared in appreciable numbers in members of the third and fourth generations. The tumors develop earlier in the members of strain 38^3 than in the members of strain 43. Note that the members of strain 38^3 have been produced by three generations of brother-to-sister matings.

The mating procedure followed may be illustrated by tracing the history of tumorous fish 38^3. In the mating of 33-5 and 33-15 the female and male were non-tumorous but some of their siblings were tumorous. To diagram this mating the horizontal line connects the white areas of the circle and the square. In the next mating, below, a tumorous female (38-1) was mated to a non-tumorous male (38-11). In this instance, the horizontal line connects the black area of the circle to the white area of the square.

cells increased as the thyroid tissue become more abnormal. The standard errors were lowest in the series of measurements of the smallest cells; hence, these are the measurements which were used.

The distribution of the thyroid tumors between the sexes was noted. The length of each fish was recorded as the distance between the anterior tip of the snout and the end of the urosome, to determine whether a relationship exists between the size of the fish and stage of development of the tumor.

RESULTS

HISTOLOGY OF THE THYROID TISSUE FROM WILD MONTEZUMA SWORDTAILS PRESERVED AT THEIR SITE OF CAPTURE

Fourteen wild swordtails (six immature, three adult females, and five adult males, fixed in 10 per cent formalin at their site of capture) were examined. The thyroid elements in the wild swordtails, as in most teleosts, were unencapsulated (Gudernatsch [10]) and consisted of isolated follicles distributed in the stroma along the ventral aorta and the bases of the first, second, and third aortic arches. They were most numerous between the first and second arches, and just anterior to the third arch; none were in the gills.

The variation in the number of follicles was great, being 13–35 in immature fish (Fig. 2), 33–75 in seven of the mature fish, and 108 in one male. The number of follicles tended to vary directly with the size of the fish ($r = +0.88$).

In thirteen of the fourteen wild fish fixed in the field, each follicle was composed of flat to low cuboidal epithelial cells, 2–4 μ in height (Fig. 2), with centrally located, elongated nuclei. In the fourteenth, an exceptional wild male with 108 follicles, the epithelial cells in several follicles were columnar. The columnar cells contained a colloid-like substance at the basal ends and nuclei at the apical ends.

In mature wild fish the epithelial cells inclosed a lumen filled with dense, sometimes granular, acidophilic colloid in which occasional epithelial cells were suspended. In the immature fish the colloid was sparse.

HISTOLOGY OF THE THYROID TISSUE OF LABORATORY-REARED SWORDTAILS OF THE FIRST GENERATION, STRAIN 21

Three adult fish from strain 21, representing the first generation of laboratory-reared Montezumas, fixed in formalin in 1940, were available for analysis. Unfortunately, after 12 years the fish were in a poorly preserved state, and their tissues did not react favorably to histological technics. The complete distribution of the thyroid follicles could not be determined, but thyroid follicular structure was similar to that of the adult wild fish fixed in Mexico.

HISTOLOGY OF THE THYROID TISSUE OF THE LABORATORY-REARED MEMBERS OF THE SEVENTH GENERATION OF THE SWORDTAILS, STRAIN 38^3

Both parents of the members of strain 38^3 had large, externally visible thyroid tumors. Fifteen of their offspring were sacrificed for a study of the development of the thyroid tumor.

Two fish were fixed on their day of birth; one had 22, the other 23, thyroid follicles. In all significant respects, the follicles resembled those of the immature wild fish fixed in the field.

Two fish were fixed 1 month after birth. They had twice as many follicles as did the 1-day-old animals. The new follicles appeared to multiply by budding from the older follicles. The epithelial cells of the follicles were columnar, 4–6 μ in height, and were significantly taller ($\frac{d}{\sigma_d} = 3.18$) than those of the wild fish. The nuclei of the epithelial cells are centrally located in one fish and basally in the other.

Five fish were sacrificed 2 months after birth. Their lengths were in the same range as those of the immature wild fish (14–21 mm.). The thyroid glands in the laboratory-reared fish had very much less stroma and many more thyroid follicles than the glands of the wild fish. The follicles extended from the first aortic arch posteriorly to the fourth aortic arch. The blood vessels and capillaries near the follicles were engorged with red blood cells, some of which lay outside the vessels. The epithelial cells were 7–9 μ high; their nuclei were eccentric in position (Fig. 3). No lumen was present in some follicles; others contained homogeneous, acidophilic colloid in scant amounts. These glands were typical of hyperactive thyroids as defined by Cowdry (4). Two mitotic figures were found in the thyroid epithelial cells of one fish; they were the only mitoses observed in all the swordtails examined.

Two fish were sacrificed at $2\frac{1}{2}$ months of age. Both contained over 125 follicles, many of which were either distorted in shape or partially disintegrated. They extended from the first to the fourth aortic arches. The follicle cells were of the same size and shape as those of the 2-month-old fish and occasionally contained a colloid-like substance.

Four fish were sacrificed at 5 months of age. Their thyroid tissue extended from the first aortic arch to the base of the ventral aorta. A few large colloid-containing follicles bounded by cuboidal or columnar epithelial cells were found near the first aortic arch, at the base of the ventral aorta, and at the ventral margin of the lower jaw. The rest of the thyroid tissue was composed of hypertrophied epithelial cells. Their nuclei were swollen and basal in position; their cytoplasm was acidophilic

and occasionally contained a granular, colloid-like substance. Some of the epithelial cells were single, others formed small groups, still others were arranged in cords or in microfollicles. The epithelial cells did not extend into the gills, or around the gill cartilage or bone, but in one animal they surrounded some of the opercular musculature (Figs. 4 and 5). The over-all histological picture was one of a solid mass of cells in a highly vascular area that was frequently hemorrhagic. Very little connective tissue stroma was present.

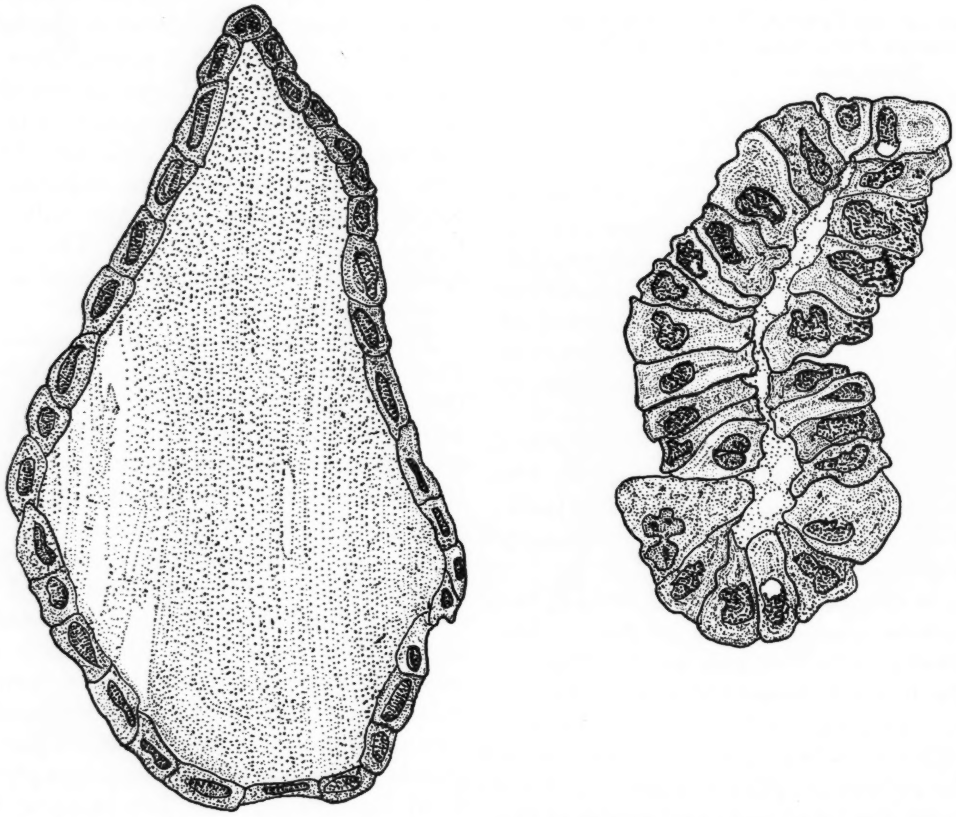

CHART 2.—Representative thyroid follicles from Montezuma swordtails. Left: inactive thyroid follicle from a normal, wild swordtail; note the low cuboidal epithelial cells at the periphery and the evenly dispersed colloid in the center. Right: hypertrophied follicle from a pretumorous, laboratory-reared swordtail; note the high columnar epithelial cells with eccentrically placed nuclei and the sparse colloid. Camera lucida drawing. Mag. ×330.

Fifteen fish, of the same brood, now $7\frac{1}{2}$ months old, are still under observation; one of them, a male, shows externally the beginning of a thyroid tumor.

Histology of the Thyroid Tissue of Laboratory-reared Swordtails of the Seventh Generation, Strain 43

The male parent of strain 43 had a small, macroscopically visible thyroid tumor; the female did not. The thyroidal tissues of two of their 5-month-old young were examined and found to be normal. Fifteen of their offspring, between 5 and 12 months of age, were fixed for the purpose of comparing the early and late developing tumors.

Five fish were sacrificed at 6–$6\frac{1}{2}$ months of age. Four of the five had 52–58 thyroid follicles with dense colloid. Their epithelial cells were either low cuboidal, high cuboidal, or columnar; they were neither swollen nor hypertrophied but for the most part were taller than those in the wild fish. (The glands apparently were highly active but not atypical.) The remaining swordtail was $6\frac{1}{2}$ months old and had 165 small, compact follicles with columnar epithelial cells.

Two fish were sacrificed at 7 months of age. One had many follicles with comparatively low epithelium and predominantly basophilic colloid. The other had very few follicles, all filled with sparse acidophilic colloid and lined by greatly hypertrophied epithelial cells with swollen nuclei. The epithelial cells of this abnormal male were compared to those of a normal swordtail in Chart 2.

Three fish were sacrificed at 10–11 months of age. They had hypertrophied and hyperplastic

tissue comparable to that of the 2½-month-old members of strain 38^3.

One fish fixed at 10 months and two others sacrificed at 12 months of age had tumorous thyroid tissues comparable to those in the 5-month-old members of strain 38^3. The first externally visible thyroid tumor appeared in a 10-month-old male.

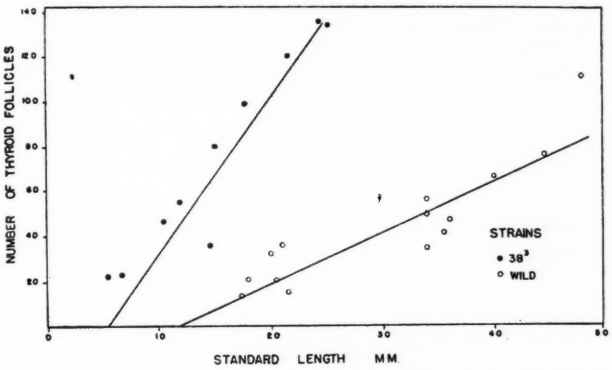

CHART 3.—The relationship between the number of thyroid follicles and the length (growth) of Montezuma swordtails. Right: white circles (○) represent normal, wild swordtails fixed at their site of capture in Mexico. Note the gentle slope of the regression line indicating that as the fish increase in length there is a gradual increase in the number of their thyroid follicles. Left: black circles (●) represent laboratory-reared, tumor-susceptible swordtails of strain 38^3. Note the steep slope of the regression line, indicating that as the fish grow there is a rapid increase in the number of their thyroid follicles.

Eight fish, six females and two males, now 17 months old, are still alive; all except two females have externally visible thyroid tumors.

NUMBER OF THYROID FOLLICLES IN RELATION TO STRAIN OF FISH

At birth both normal and tumorous Montezuma swordtails have few thyroid follicles. As the fish develop, the number of follicles increases; but there is a distinct difference in their rate of multiplication in normal and tumor-susceptible swordtails. They increase slowly in wild swordtails, quite rapidly in laboratory swordtails of strain 43 (in which tumors develop at the end of a year), and extremely rapidly in members of strain 38^3 (which become tumorous 5 months after birth).

In Chart 3 the number of follicles is plotted against the lengths of the wild fish and of the fish from strain 38^3. In Chart 4, the number of follicles is plotted against the age of the swordtails of strains 43 and 38^3. The comparisons are made as indicated, because no age records are available for the wild fish and no young swordtails of strain 43 were examined. Each of the two comparisons is valid because, from birth to sexual maturity, the age and length of poeciliid fish are positively correlated (Chavin and Gordon [3]).

The relative positions of the two regression lines and their slopes illustrate the differences in the rate of increase of the thyroid follicles in the various strains of fish. In Chart 3, the regression line for strain 38^3 is to the left and that for the wild fish to the right, indicating that young wild swordtails have fewer follicles than laboratory-reared swordtails. The slope of the left regression line is much steeper than that of the right, showing that the increase in the number of thyroid follicles is more rapid in pretumorous than in wild swordtails.

Furthermore, Chart 4 shows that the increase in the number of thyroid follicles is more rapid in those swordtails that will develop tumors after 6 months than in those that do not develop tumors until they are 1 year old.

MACROSCOPIC APPEARANCE OF THE THYROID TUMOR IN SWORDTAILS OF VARIOUS AGES

Thyroid tumors were detected externally in 27 of the 34 members of strain 38^2, which has been inbred for six generations. The first tumor appeared

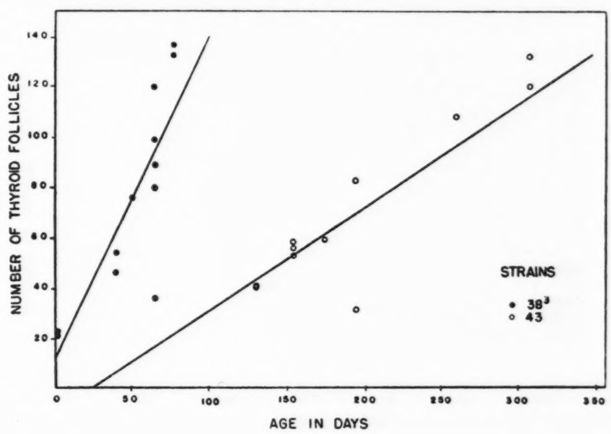

CHART 4.—The relationship between the number of thyroid follicles and the age of Montezuma swordtails. Right: white circles (○) represent laboratory-reared swordtails of strain 43. (Members of this strain develop tumors when 12 months old.) Note the slope of the regression line indicating that as the fish age there is a fairly rapid increase in the number of their thyroid follicles. Left: black circles (●) represent laboratory-reared swordtails of strain 38^3. (Members of this strain develop tumors when 6 months old.) Note the steep slope of the regression line indicating that as the fish age there is a more rapid increase in the number of their thyroid follicles.

in a 24-month-old female swordtail. Ten months later 27 of the 34 fish had visible thyroid tumors (Table 1). Four of the remaining seven, without visible tumors, were dissected. Each had a small tumor at the base of its tongue.

The growth of one tumor was closely observed after it was first detected as a small, pale, almost invisible lump in a 30-month-old female. At 34

months the tumor extended beneath the opercula where it grew rapidly and spread to the gill region. At 35 months the female became moribund and was sacrificed. Her right operculum was dissected away, exposing a large, firm, white tumor that projected from the gills to the heart, was almost as wide as the body cavity, and was $\frac{3}{4}$ the size of the head (Chart 5). This tumor, one of the largest in this species, is noteworthy, because it grew to its large size in less than 5 months.

TABLE 1

AGE AT WHICH THYROID TUMORS IN 34 MEMBERS OF THE SIXTH GENERATION INBRED STRAIN, 38², OF THE SWORDTAIL *X. montezumae* WERE DETECTED EXTERNALLY

Age (months)	Females Tumor	Females No tumor	Males Tumor	Males No tumor
24	1	25	0	8
29	13	13	2	6
34	22	4	5	3

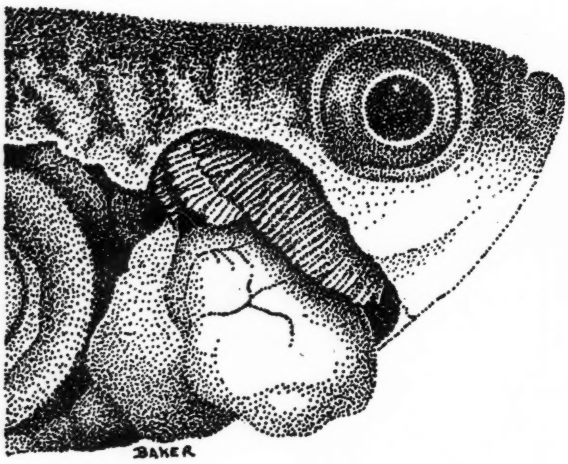

CHART 5.—An adult female Montezuma swordtail showing a large thyroid tumor in the region of the gills which developed in less than 5 months. The tumor is $\frac{3}{4}$ of the size of the head and extends from the gills to the region of the heart. Mag. ×4.

POSSIBLE GENETIC LINKAGE

Since the development of thyroid tumors in swordtails is evidently under some polygenic control, it seemed possible that some of those genes might be linked to some of the known color pattern genes. The Montezuma has two dominant genes that control the distribution of macromelanophores or large black pigment cells on the body: *Sc* for macromelanophore spotting in the caudal fin, *Sp* for similar spots on the body proper. The recessive is indicated as + and denotes the absence of macromelanophores. The macromelanophore pattern of each fish was recorded in broods of strain 38² (Table 2). The analysis demonstrated that there was no linkage between the presence of *Sp*, *Sc*, or the recessive (+) genes and the presence of thyroid tumors.

TABLE 2

THYROID TUMOR INCIDENCE IN MEMBERS OF THE SIXTH GENERATION INBRED STRAIN, 38², OF THE SWORDTAIL *X. montezumae*, 28 MONTHS OLD

Macromelanophore pattern	Females Tumor	Females No tumor	Males Tumor	Males No tumor
Spotted-caudal	4	1	0	1
Spotted-sides	4	7	1	4
None	5	5	1	1
Total	13	13	2	6

DISCUSSION

The observations of Gaylord and Marsh (5) on the development of thyroid tumors in hatchery-reared trout may be compared to those of Aronowitz (Berg), Edgar, and Gordon (1) on the development of similar tumors in laboratory-reared swordtails. The salient comparative details are presented in Table 3. Four changes—namely, hypertrophy of follicle cells, hyperplasia of follicles, reduction of colloid within the follicles, and hyperemia—were characteristic of both tumors. Hyperplasia of the connective tissue around the follicles was found in the trout but not in the swordtail.

In both kinds of wild fishes hypertrophy and hyperplasia of the follicles are occasionally found, but actual thyroid tumors, although rare, have been found only in wild trout (Table 3).

Gorbman and Gordon (8) stated that one seemingly significant difference between the thyroid tumors of the swordtails and those of trout is the age at which they develop. In trout the tumors are relatively large in *young* animals, but they later diminish or regress in some older animals. On the other hand, the growths appear at maturity in the fifth generation laboratory-reared swordtails and grow until death intervenes. The tumors in the seventh generation swordtails are fully formed by 5 months. Apparently, tumors develop earlier in members of the more highly inbred tumor-bearing strains, suggesting that the time of tumor development depends to some degree upon their genetic constitution.

The Montezuma represents one of the seven species of xiphophorin fishes that have been maintained by inbreeding for many generations under practically identical environmental conditions. In only two species, *montezumae* and *pygmaeus*, the majority of a brood becomes tumorous. Gorbman

and Gordon (8) suggested that the laboratory-reared Montezumas may have an extremely high iodine requirement—one which exceeds the ordinary dietary supply. They pointed out, however, that the dried ocean shrimp, which composes a large part of their diet, has a high concentration of iodine. It is worth noting that Nigrelli (13) and Schlumberger and Lucké (15) have reported that several species of *marine* fishes maintained under aquarium conditions develop thyroid tumors.

In following up the lead on a high iodine requirement, two series of experiments are now in progress: (*a*) the raising of young swordtails in iodized water of known concentration and (*b*) the treating of tumorous animals with potassium iodide, thyroxine, or whole dessicated thyroid. Preliminary results indicate that the tumors regress under these treatments and that potassium iodide has a prophylactic effect.

The incidence of the thyroid tumor is 100 per cent only in the most highly inbred strains of Montezuma swordtails, suggesting that the incidence of tumors may be related to polygenes which become homozygous upon inbreeding. Since thyroid hyperplasia is found more frequently in many species of fishes living under aquarium conditions than in the wild state, the role of the environment, particularly the quality of the water, will have to be carefully evaluated and correlated with the genetic influences.

The basic similarity between the thyroid follicles of all vertebrates has been stressed by Goldsmith (6). According to Bullock and Curtis (2) and Slye, Holmes, and Wells (16), spontaneous thyroid carcinomas are found in the primate mammals and are rare in all nonprimate and nonmammalian vertebrates. These authors take the view of Marine and Lenhart (11, 12) rather than that of Gaylord and Marsh (5) and regard the thyroid tumor of the trout as a severe endemic goiter rather than a true carcinoma. It is difficult to differentiate between cancerous and benign thyroid tumors in trout and swordtails where the two criteria, invasiveness and metastasis, are not applicable, because (*a*) the thyroid tissue is not encapsulated, and the point at which invasiveness begins cannot be precisely determined, and (*b*) the tumors rarely metastasize even when highly malignant.

The developmental pattern of the swordtail thyroid tumor may be profitably contrasted with the developmental pattern of malignant human thyroid tumors. According to a review by Gorbman (7), the most malignant thyroid tumors in man originate from hypoplastic glands. The growth of tumors in the swordtail and the trout was preceded by hyperplastic growth of the thyroid tissue. Therefore, the fishes' pattern of development is more typical of endemic goiter than of true carcinoma. This inference is strengthened by the knowledge that the thyroid tumors of fishes regress when treated with iodine or thyroxine.

TABLE 3

CHARACTERISTICS OF NORMAL AND TUMOROUS THYROIDAL TISSUE IN SPECIES OF WILD AND DOMESTICATED TROUT AND MONTEZUMA SWORDTAILS

PRETUMOROUS CHANGES	TUMOR	TROUT Wild	TROUT Hatchery	SWORDTAILS Wild	SWORDTAILS Laboratory "43"	SWORDTAILS Laboratory "38^3"
Hypertrophy of follicle cells		occasional	extreme	occasional	extreme	extreme
Reduction of colloid in follicle		normal	extreme	normal	extreme	extreme
Hyperplasia		occasional	extreme	occasional	extreme	extreme
Hyperemia		absent	present	absent	present	present
Hyperplasia of connective tissue		absent	present	absent	absent	absent
	incidence	very rare	endemic at one time	not seen*	100 per cent	100 per cent
	time of development	unknown	by 4 months		by 6 months	by 1 year
	time of regression		after 3 years		never has regressed in any†	never has regressed in any†

* Among approximately 1,000 wild swordtails examined.

† Some are 3 years old, almost the maximal attainable age.

SUMMARY

The progressive stages in the development of the thyroid tumor of the swordtail, *Xiphophorus montezumae*, are as follows: 1. Day-old fish have 20–30 thyroid follicles, lined by flat or low cuboidal epithelium, scattered in the stroma around the ventral aorta, but not in the gills. 2. The follicle cells increase in size and become high columnar. 3. The number of thyroid follicles increases; the blood capillaries in the region of the follicles become and remain engorged with red blood cells. 4. The epithelial cells of the new follicles simul-

taneously increase in number and size; some of the blood capillaries rupture, and individual red blood cells are found close to the follicular cells. 5. The follicle configuration becomes distorted. 6. The follicles disintegrate. 7. Tumorous growths develop composed primarily of a mass of epithelial cells, microfollicles and hemorrhages. 8. Muscles in the region of the ventral aorta are surrounded by tumorous afollicular epithelial cells. 9. Tumor cells destroy the deeper musculature, the gill filaments, cartilage, and bone. (At this stage the thyroid tumor is visible externally.) 10. Death may be due to destruction of visceral gill arches and the consequential interference of normal respiration.

Highly inbred fish, from brother-to-sister matings between members of a thyroid tumor-susceptible strain, develop tumors earlier in life than those from less intensely inbred strains. This suggests that genes influencing the growth of thyroid cell elements accumulated in the members of the inbred strains.

A comparison was made between the thyroid follicles of wild young fish taken directly from their natural habitat in Mexico, and young fish laboratory-reared for seven generations. In both wild and laboratory fishes the number of follicles increase with age and length, but the increase is far more rapid in the laboratory fishes.

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Fig. 1.—Thyroid tumors in two adult laboratory-reared *Xiphophorus montezumae:* male, above, with spotted (*Sp*) pattern; female, below, with spotted caudal (*Sc*) pattern. The tumor in the male is visible below the jaw. In the female the opercula are forced away from the head by tumor tissue. Mag. $\frac{3}{4}$ normal size.

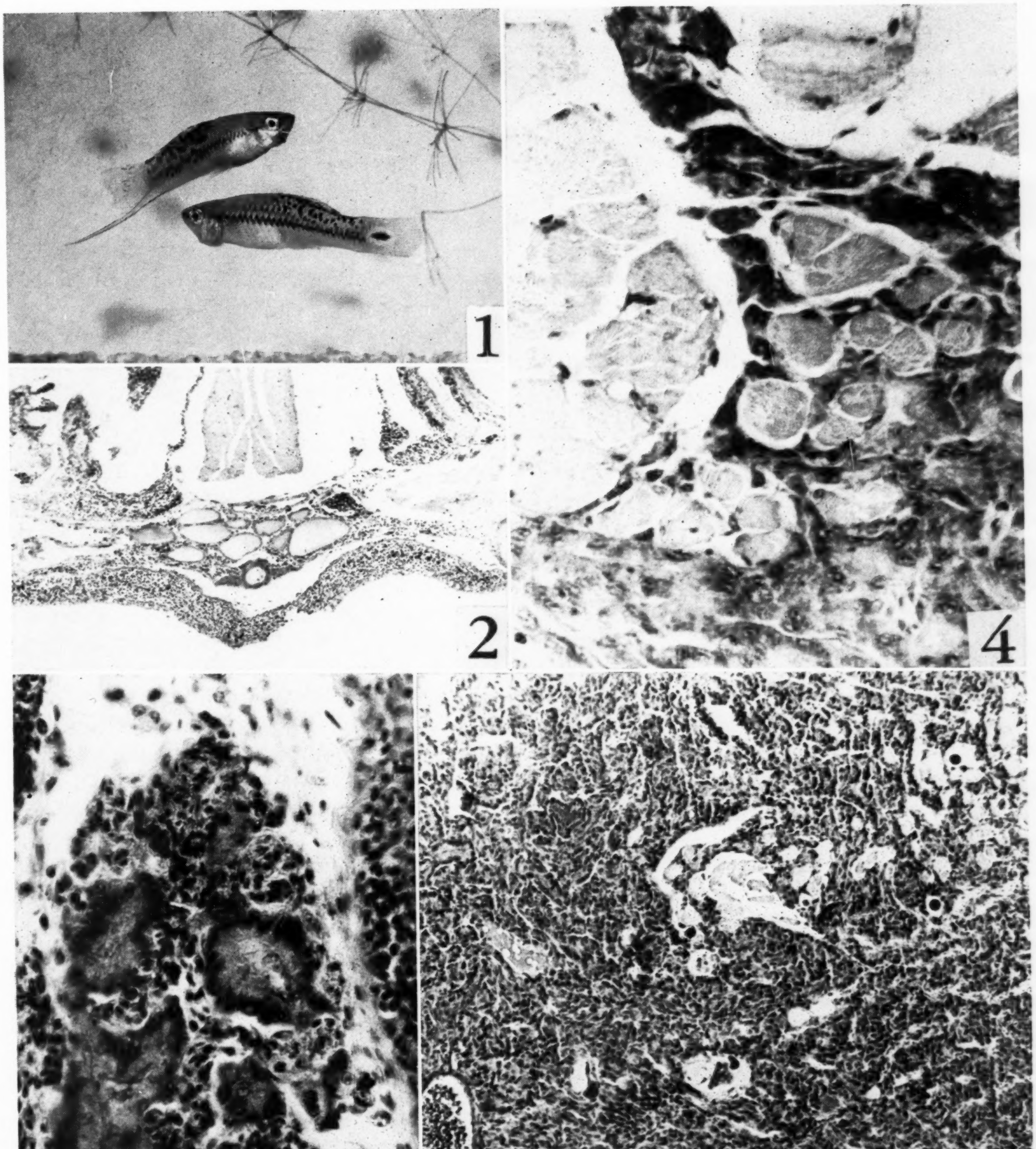
Fig. 2.—Cross-section through the lower jaw of a wild, immature Montezuma swordtail, preserved at its site of capture in Mexico. Note the unencapsulated, normal thyroid follicles scattered in the stroma between the branchial musculature and blood vessels. Hematoxylin-eosin. Mag. ×130.

Figs. 3–5.—Sections through the lower jaws of laboratory-reared swordtails of strain 38^3. All sections are stained with hematoxylin-eosin.

Fig. 3.—From a 2-month-old swordtail. Note the absence of lumina and the extreme vascularization of the stroma. Mag. ×510.

Fig. 4.—From a 5-month-old male swordtail. This thyroid tumor was revealed by histological methods. Note the beginning of muscle invasion by tumor cells. Mag. ×440.

Fig. 5.—From a 5-month-old male swordtail. This thyroid tumor was revealed by histological methods. Note the absence of follicular structure and the cordlike arrangement of cells. Mag. ×143.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An Attempt To Induce Resistance in an Inbred Strain of Mice by Ligation of a Homologous Tumor*

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It was observed by Allen (1) that occlusion of the blood vessels of neoplasms in man and in some of the lower animals for 3–12 hours damaged malignant tissues more than normal tissues. Later, Lewis and Aptekman (10, 11) found that, by employing a similar procedure, they could induce in an inbred strain of rats a resistance to a sarcoma indigenous to the strain.

In our laboratory, attempts were made after the manner of Lewis and Aptekman (10) to induce, in an inbred strain of animals, resistance against a tumor homologous to the strain by causing an initial tumor implant to atrophy through occlusion of its vascular supply. Our investigations, with an inbred strain of mice instead of rats, failed to confirm the work of Lewis and Aptekman, although several procedures were employed in an effort to induce tumor resistance.

In our preliminary investigations the findings were for the most part negative, and in some cases inconsistent. This report presents the results of a series of experiments designed particularly to attempt to trace some of the inconsistencies encountered.

EXPERIMENTAL PROCEDURE

A total of 147 DBA/1 female mice (4–5 weeks of age), obtained from the Roscoe B. Jackson Memorial Laboratory, was employed in these experiments. The transplantable mammary carcinoma dbrB, homozygous to the strain, was used in all experiments. Sixty mice (with the exception of the controls and three groups of "recipients") were given a subcutaneous inoculation of 0.1 cc. suspension of 1 part of minced tumor tissue to 9 parts of 0.9 per cent normal physiological solution. Directly after inoculation, the mice were housed in individual cages and separated into four groups of fifteen mice. Tumor volumes were determined (three dimensions with calipers) 7, 9, and 12 days postinoculation. Twelve days postinoculation, the tumors of all mice were ligated by lifting the tumors and the skin and tying several loops of strong thread around the bases of the tumors. In this manner the tumor was confined in a tightly closed pocket of skin.[1] Twenty-four hours later, the ligatures were removed from the tumors of one series of mice (group A). Each atrophied tumor was excised under aseptic conditions and divided into two equal parts. One fragment was used for auto-implantation (group A). The other fragment was transplanted (homotransplant) to a "recipient," in fifteen mice constituting group B (each donor and recipient was identified for future comparison). The ligatures were removed from the tumors of fifteen additional mice after 48 hours, and half of the excised tumors were used for auto-implant (group C) and homotransplant (group D). The ligatures were removed from another fifteen mice after 72 hours, and similar auto-transplants (group E) and homo-transplants (group F) were performed. In another series of mice (group G), the tumors were ligated "permanently." When the auto-transplants, homotransplants, and "permanently" ligated devitalized tumors had been in part or completely resorbed in their hosts (23 days postligation), all groups, including the control group I, were given subcutaneous inoculations of viable dbrB tumor suspension in amounts equal to the original inoculations. (Mice in group H, which were also "permanently" ligated, were not given a challenge dose, but were merely kept for observation.) Thereafter, volumes of the "test" or "challenge" tumors were determined after 7, 9, 12, and 15 days postinoculation.

The preparation and inoculation of tumor suspension, the ligation of tumors, and the measurement of tumor volumes were performed by one

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[1] During the course of this work, the ligation technic was employed independently by Zahl (16).

person in order to obviate as much as possible variations in technic.

EXPERIMENTAL RESULTS

It will be noted from an inspection of Tables 1 and 8, that the volumes of the tumors in groups A, C, E, G, and H exhibited good uniformity whether measured on the seventh, ninth, or twelfth day following inoculation of the original viable tumor suspension. It was thus indicated that the tumor employed was reasonably homogeneous, the strain comparatively pure, and the inoculation technic fairly consistent.

Table 2 shows the response of groups A and B to the test tumor inoculation given 23 days post-ligation. Comparing group A, in Table 1, to group A, in Table 2, it will become apparent at once that the auto-transplantation of 24-hour ligated tumors did not promote resistance against the challenge tumor. The uniformity in response to viable tumor inoculation persisted (compare averages of 0.08, 0.5, 1.34 of group A in Table 2 to averages of 0.07, 0.29, 1.58 of group A in Table 1). Similarly, when the volumes of the test tumors of group B (recipients) are compared to those of group A (donors) in Table 2, it again becomes evident that no resistance was established by prior implantation of devitalized tumor tissue.

Tumors that had been submitted to 48 and 72 hours, and "permanent" ligation also fail to demonstrate any degree of immunity as a consequence of the treatment. (Compare the experimental groups in Tables 3–5 to the control group I in Table 6).

TABLE 1

VOLUME OF TUMORS (CC.) PRIOR TO LIGATION

Mouse no.	Group A: 7	Group A: 9	Group A: 12	Mouse no.	Group C: 7	Group C: 9	Group C: 12
	Days posttranspl. — Tumor volume (cc.)				Tumor volume (cc.)		
313	.06	.22	1.69	336	.05	.23	1.80
317	.05	.14	1.98	337	.10	.48	2.69
318	.13	.38	1.69	338	.07	.50	2.29
319	.08	.21	1.82	339	.10	.94	1.30
320	.04	.12	1.08	341	.00	.72	1.87
321	.06	.29	2.10	342	.02	.21	1.10
322	.12	.22	1.82	343	.08	.48	1.39
323	.07	.11	1.19	344	.08	.18	1.40
325	.03	.09	1.20	346	.10	.48	3.28
326	.07	.16	1.72	347	.14	.80	1.20
327	.06	.07	1.19	352	.16	.60	1.19
328	.08	.97	1.51	353	.05	.38	1.44
329	.09	.26	.80	354	.09	.72	.96
332	.06	.56	1.87	356	.12	.40	1.45
333	.07	.55	2.11	357	.08	.29	1.56
Av.	.07	.29	1.58		.08	.49	1.66

Mouse no.	Group E: 7	Group E: 9	Group E: 12	Mouse no.	Group G: 7	Group G: 9	Group G: 12
358	.18	.60	1.87	390	.02	.83	1.92
359	.10	.25	1.64	391	.28	.24	1.40
361	.06	.45	1.17	392	.25	.72	1.57
362	.08	.75	2.86	393	.09	.45	1.72
364	.06	.28	.77	396	.07	.56	1.08
366	.02	.29	.99	398	.07	.28	1.68
368	.16	.65	1.68	399	.07	.48	2.08
369	.04	.36	1.70	402	.02	.12	1.65
372	.14	.72	1.60	403	.02	.35	1.54
373	.16	.78	3.32	404	.07	.58	1.04
374	.07	.59	2.18	405	.07	.28	.96
380	.12	.63	2.11	406	.06	.50	2.31
382	.15	.56	1.23	411	.14	.54	1.60
383	.14	.68	1.98	412	.12	.53	2.24
386	.12	.72	2.31	413	.14	.40	2.24
Av.	.11	.55	1.83		.10	.46	1.67

TABLE 2

VOLUME OF TEST TUMORS (CC.) AFTER IMPLANTATION WITH 24-HOUR LIGATED TUMORS

Mouse no.	Group A (auto-transplants): 7	9	12	15	Mouse no.	Group B (homo-transplants): 7	9	12	15
	Days posttranspl. — Tumor volume (cc.)					Tumor volume (cc.)			
313	.00	.31	.94	1.80	503	P	.13	1.68	Died
317	.12	.49	1.08	1.35	504	.12	.42	1.07	Died
318	.03	.24	1.12	2.80	505	.02	.54	.84	Died
319	.14	.50	1.60	1.76	506	.10	.65	1.36	2.80
320	.02	.26	.90	1.71	507	.26	1.12	2.09	Died
321	.09	.55	1.76	1.70	508	.12	.28	.93	2.60
322	.06	.42	1.94	1.44	509	.15	.28	1.36	1.48
323	.11	.84	1.62	1.62	510	.13	.32	1.50	1.87
325	Died				511	Died			
326	.06	.36	1.22	2.40	512	.06	.46	1.36	3.70
327	.00	.24	.68	2.40	513	.13	.82	1.70	1.78
328	.09	.78	1.35	Died	514	.06	.54	1.26	2.34
329	Died				515	.11	.36	1.35	Died
332	Died				516	.14	.32	1.39	Died
333	.20	1.01	1.87	1.87	517	.14	.48	1.68	2.16
Av.	.08	.50	1.34	1.90		.12	.48	1.39	2.30

P = palpable.

In comparing the history of the "permanently" ligated tumors (Table 7, group G) to the volumes of the test tumors in the same group (Table 5), it will be observed that no relationship exists between the behavior of the ligated tumor and the host's response to the test inoculation. Thus, whether the ligated tumor remains present, partially present, or was lost early or late over the observation period exercised little if any effect on the host's susceptibility or resistance.

The relative volumes of the tumors in all groups are given in Table 9, which clearly indicates no significant difference between the first and second inoculation of viable tumor tissue, nor between the 24-hour, 48-hour, 72-hour, and "permanent" ligation groups.

DISCUSSION

Although it has been demonstrated repeatedly by many investigators (1, 2, 4, 6–10) that inbred animals cannot be immunized with homologous or neoplastic tissue against a tumor which originated in the strain, some have reported (8, 9) that, under certain experimental procedures, resistance can be induced against a tumor homologous to its native strain. Since it has been shown (3, 5, 11–13) by transplantation studies that certain tumors can undergo a "change" in their transplantation pattern which is accompanied by certain physiologic and genetic differences, it might be suggested that either failure or success in immunization experiments depends, at least in part, upon the "changed"

TABLE 3

VOLUME OF TEST TUMORS (CC.) AFTER IMPLANTATION WITH 48-HOUR LIGATED TUMORS

Mouse no.	Group C (auto-transplants) 7	9	12	15	Mouse no.	Group D (homo-transplants) 7	9	12	15
	Days posttranspl.; Tumor volume (cc.)					Tumor volume (cc.)			
336	P	.02	.38	1.94	518	.00	.62	1.22	1.76
337	.10	.42	1.01	Died	519	.10	.21	.86	1.29
338	.01	.25	1.02	1.88	520	.17	.91	1.70	Died
339	.12	.33	.59	1.62	521	.20	.88	1.72	1.98
341	.28	.59	1.09	2.47	522	.16	.64	1.30	2.20
342	.08	.49	1.76	2.38	523	.09	.46	1.98	2.86
343	.07	.24	.98	2.43	524	.20	1.16	1.92	Died
344	.11	.38	1.54	2.94	525	.04	.46	1.68	2.28
346	P	.24	1.65	2.38	526	.00	.31	1.07	1.98
347	Died				527	P	.10	.34	1.56
352	.02	.27	1.58	2.06	528	.04	.13	.50	1.70
353	.14	2.81	1.92	Died	529	.06	.38	1.13	1.62
354	Died				530	.13	.34	1.12	1.58
356	Died				531	.10	.62	1.20	1.36
357	.24	.84	1.78	2.20	532	.11	.59	1.60	Died
Av.	.12	.57	1.27	2.20		.10	.52	1.29	1.80

P = palpable.

TABLE 4

VOLUME OF TEST TUMORS (CC.) AFTER IMPLANTATION WITH 72-HOUR LIGATED TUMORS

Mouse no.	Group E (auto-transplants) 7	9	12	15	Mouse no.	Group F (homo-transplants) 7	9	12	15
	Days posttranspl.; Tumor volume (cc.)					Tumor volume (cc.)			
358	.00	.06	.50	1.82	533	.04	.55	1.87	Died
359	.12	1.40	1.26	1.12	534	.28	.54	1.15	1.71
361	.01	.45	1.76	2.24	535	.14	.50	.90	1.98
362	.19	.57	3.23	2.64	536	.02	.22	.59	1.44
364	Died				537	.04	.55	1.44	3.30
366	Died				538	Recurrence			
368	.10	.32	1.68	2.38	539	.28	.98	1.19	4.22
369	.01	.13	.94	3.38	540	.06	.42	1.01	1.63
372	.08	.53	1.36	3.38	541	.22	.81	1.58	2.52
373	.02	.33	1.01	2.40	542	.12	.36	1.20	1.48
374	.26	.68	2.16	1.94	543	P	.11	.45	1.73
380	.06	.46	1.65	2.14	544	.02	.33	1.30	2.64
382	.02	.32	1.20	3.00	545	.01	.49	.78	1.84
383	.11	.62	1.73	1.97	546	.22	.63	1.85	Died
386	.08	.52	1.68	1.99	547	.13	.50	1.52	Died
Av.	.08	.49	1.55	2.30		.11	.50	1.34	2.20

genetic constitution of the tumor in relation to the constitution of the host strain. Thus, the successful induction of immunization against certain transplantable tumors may require that the "change" be in the direction from a state of high specificity (multi-factorial) to one of low or nonspecificity. It was observed by Strong (12), for example, that a tumor homologous to a particular inbred strain, after many transplant generations, grew with considerably more rapidity than did its predecessors. Transplantation experiments showed that this "changed" tumor could be successfully grown in all F_2 individuals of the BALB-DBA cross. It also grew in several back-cross generations toward the original nonsusceptible stock, in all members of the nonsusceptible stock, and in all mice, regardless of their genetic relationships.

It is not impossible that in one case an immunity can be established against a tumor homologous to its strain of origin, while in another case, when certain and slight genetic differences do not

TABLE 5

VOLUME OF TEST TUMORS (CC.) AFTER IMPLANTATION WITH PERMANENTLY LIGATED TUMORS

GROUP G

Mouse no.	Days posttranspl. 7	9	12	15
	Tumor volume (cc.)			
390	.00	.18	.97	1.92
391	.19	.35	1.35	1.61
392	.00	.06	.14	1.09
393	.20	.97	1.44	1.80
396	.05	.60	2.26	Died
398	.09	.98	1.87	2.05
399	.08	.42	1.40	3.00
402	.03	.27	1.40	1.83
403	.01	.18	.65	1.50
404	.08	.56	1.26	Died
405	.03	.55	1.50	2.04
406	.04	.77	1.35	1.54
411	.07	.65	1.22	1.62
412	.36	.94	2.20	Died
413	.08	.42	1.28	2.28
Av.	.08	.53	1.35	1.80

TABLE 6

VOLUME OF CONTROL TUMORS (CC.) FOR GROUPS A, B, C, D, E, F, AND G

GROUP I

Mouse no.	Days posttranspl. 7	9	12	15
	Tumor volume (cc.)			
548	.09	.44	1.43	1.68
549	.15	1.07	1.71	1.76
550	.11	.76	1.22	2.20
551	.01	.49	1.23	1.76
552	.19	.98	2.25	2.57
553	.10	.59	1.53	2.16
554	.09	.36	1.53	2.16
555	.02	.39	1.15	1.90
556	.05	.82	1.73	1.73
557	.10	1.23	1.35	Died
558	.05	.76	1.12	1.98
559	.08	1.01	1.53	2.60
560	P	.19	.42	1.36
Av.	.08	.70	1.40	1.80

P = Palpable.

TABLE 7

HISTORY OF TUMORS PERMANENTLY LIGATED

GROUP G

Mouse no.	Days postligation 4	7	10	12	16
390	T	T	T	T	—
391	T	t	t	—	—
392	T	T	T	T	T
393	—	—	—	—	—
396	T	T	T	T	—
398	t	—	—	—	—H
399	T	t	t	t	t
402	T	t	t	—	—H
403	T	T	T	T	—
404	—	—	—	—	—H
405	—	—	—	—	—H
406	T	T	T	T	—
411	t	t	t	t	R
412	T	—	—	—	—H
413	T	T	T	T	T

T = tumor present.
t = tumor partially present.
— = tumor lost.
R = recurrence.
H = healed.

TABLE 8

VOLUME OF TUMORS (CC.) PRIOR TO LIGATION

GROUP H

Mouse no.	Days posttranspl. 7	9	12
	Tumor volume (cc.)		
387	.10	.60	1.44
388	.14	.54	1.30
389	.21	.94	1.56
394	.11	.50	2.50
395	.06	.66	.90
397	.11	.73	2.86
400	.05	.17	.96
401	.07	.38	1.33
407	.14	.54	1.60
408	.02	.26	1.44
410	.18	.86	2.43
414	.06	.40	2.02
415	.12	.77	1.43
416	.10	.34	2.04
417	.09	.62	1.98
418	.10	.43	1.57
419	.20	.63	1.85
420	.05	.24	1.44
421	.12	.55	1.68
422	.09	.65	1.86
423	.12	.72	2.56
424	.14	.48	2.35
425	.01	.04	.51
426	.15	.58	2.24
427	.05	.19	.86
428	.05	.22	1.85
429	.08	.48	1.82
Av.	.10	.50	1.70

TABLE 9

COMPARATIVE TUMOR VOLUMES IN ALL GROUPS

		Inoculation					
		1st	2d	1st	2d	1st	2d
		Days post-inoculation					
		7	7	9	9	12	12
GROUP		Tumor volume (cc.)					
	24-hr. ligation						
A	Auto-trans.	.07	.08	.29	.50	1.58	1.34
B	Homo-trans.		.12		.48		1.39
	48-hr. ligation						
C	Auto-trans.	.08	.12	.49	.57	1.66	1.27
D	Homo-trans.		.10		.52		1.29
	72-hr. ligation						
E	Auto-trans.	.11	.08	.55	.49	1.83	1.55
F	Homo-trans.		.11		.50		1.34
G	Permanent ligation	.10	.08	.46	.53	1.67	1.35
H	Permanent ligation (no test inoculation)	.10		.50		1.70	
I	Control	.08		.70		1.40	
	Av.	.09	.10	.50	.51	1.64	1.36
	S.D.	±.01	±.01	±.12	±.03	±.13	±.09

exist between the tumor and strain, the resistant state cannot be elicited by the methods employed at present.

Since there appears to be sufficient evidence on the one hand to show that resistance can be induced in a strain against its homologous tumor, and, on the other hand, that, by employing a different tumor and strain, resistance cannot be induced, it becomes apparent that further research is required to detect the possible presence of delicate antigenic differences between malignant and normal tissue.

SUMMARY

1. Tumors ligated for a period of 24, 48, or 72 hours, when auto-transplanted or homo-transplanted, failed to protect the hosts from a challenge dose of viable tumor of the same kind.

2. Tumors "permanently" ligated failed to induce resistance against a second inoculation of viable tumor.

3. No relationship was found to exist between the rate of resorption of the ligated tumors and the subsequent resistance or susceptibility of the hosts to a second inoculation of viable tumor.

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The Effect of Nitrogen Mustard on the Cellular Concentrations of Nucleic Acids in Regenerating Rat Liver*

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Numerous publications during the last 6 years have indicated the importance of the nitrogen mustards in clinical and experimental cancer chemotherapy (9, 10, 21, 32, 33, 41, 42). The principal application has been to the treatment of lymphomas and leukemias. Two compounds, methylbis(β-chloroethyl)amine (HN2) and tris-(β-chloroethyl)amine, have been most widely used.

An equally impressive number of investigations have been concerned with some aspect of the mechanism of action of nitrogen mustard. A wide variety of cytotoxic, antimitotic, mutagenic, carcinogenic, and radiomimetic effects have been described (4, 16, 18, 20, 22, 32). From the biochemical point of view, these findings have led to a consideration of the effects of nitrogen mustard on nucleic acid metabolism. Bodenstein and Kondritzer (3) determined the nucleic acid content of successively older amphibian embryos and found that treatment of the embryos with nitrogen mustard completely prevented the normal rise of desoxyribonucleic acid (DNA) content with age but failed to affect the corresponding increase of ribonucleic acid (RNA) content. Lowrance and Carter (35) found depressions in the content of RNA and DNA and in the incorporation of P^{32} into the DNA fraction of bone marrow, spleen, and thymus in rabbits during the early periods after treatment with nitrogen mustard. Skipper *et al.* (40) demonstrated that the administration of carcinostatic agents, including nitrogen mustard, to mice reduced the incorporation of formate-C^{14} into the combined nucleic acid purines of viscera. Goldthwait (23) recently found that HN2 decreased the incorporation of both formate-C^{14} and adenine-N^{15} into adenine of DNA of rat intestine to approximately the same extent. In a recent review (16), Dustin presented the general conclusion that radiomimetic substances produce their effect by inhibiting the synthesis of DNA without causing an appreciable inhibition of the synthesis of RNA. Inhibition of nucleic acid synthesis has been proposed as the principal mechanism of action for mustard gas (25) and for x-radiation (1, 26, 29, 36, 39).

In most of these investigations the rate or extent of nucleic acid synthesis was assessed by determining the rate of incorporation of an isotopically labeled precursor. It was of interest to correlate these findings with determinations of the actual levels of both nucleic acids in the "average cells" of tissues with and without nitrogen mustard treatment. It was anticipated that an effective inhibition of synthesis would be reflected in decreased or unchanged concentrations of nucleic acids per cell. The few previous observations on the effect of nitrogen mustard on the content of nucleic acids in various tissues were made on the basis of wet weight of tissue; the recent recognition of changes in the cellularity of tissues under various conditions (28, 37)[1] indicated that analytical data must be expressed on a cell basis to permit adequate interpretation.

Various biological systems characterized by a high mitotic index and a rapid rate of synthesis of nucleic acids may be suitable for studies of this type. Technical considerations militated against the use of a mixture of viscera, as employed by Skipper *et al.* (40), for a study requiring this determination of cellularity; to speak of an "average cell" in a homogenate containing all the tissues

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[1] E. Hirschberg and A. Gellhorn, manuscript in preparation. "Cellularity" is employed throughout this paper to denote the number of cells present in a unit weight of tissue. All pertinent data have been expressed here as "number of cells or nuclei per mg. wet weight of tissue."

constituting this mixture would be meaningless. However, following the work of Higgins and Anderson (27) and Brues and collaborators (5, 6), regenerating rat liver has been widely employed as a model of rapid, non-neoplastic cell proliferation. Landing *et al.* (34) obtained an inhibition of mitosis in this tissue by nitrogen mustard but presented no data on concomitant biochemical changes. In untreated animals Stowell (43) demonstrated an increase in the nucleic acid concentration in the nucleolus and in the cytoplasm adjacent to the nucleus on the second day following partial hepatectomy, during the time of rapid cell division. The changes in the concentrations of nucleic acids per cell in the early period of liver regeneration were further defined and placed on a quantitative basis by Price and Laird (37). In the present investigation the observations of Price and Laird have been confirmed and extended to include the effect of nitrogen mustard on the cellular concentrations of nucleic acids during the period of liver regeneration. These experiments had a twofold purpose: (*a*) to evaluate regenerating rat liver as a model system for studies on the mechanism of action of cancer chemotherapeutic agents and (*b*) to subject the postulated correlation of inhibition of mitosis and inhibition of nucleic acid synthesis to a different experimental test.

METHODS

Male and female albino rats of the Wistar strain were obtained from our own stock. Animals selected for operation weighed between 150 and 200 gm. Prior to operation the rats were maintained on Purina Laboratory Chow and water ad libitum and were housed in a cooled room at a maximum temperature of 70° F. Following anesthesia with a minimal amount of ether, the rats were partially hepatectomized according to the technic of Higgins and Anderson (27).

Nitrogen mustard (methylbis(β-chloroethyl)amine hydrochloride) (HN2) was administered to the appropriate groups of animals by a single subcutaneous injection within 1 hour after the operation at a dosage level of 1.9 mg/kg. Following the operation, the rats were returned to the cooled room; they received no food except a 20 per cent solution of sucrose ad libitum. About 24 hours after the injection, a leukocyte count on tail blood was performed on every animal to assess the biological effect of HN2.

All animals were killed with ether. In the first series of experiments, rats which had received no HN2 were sacrificed at 12, 15, 24, 36, 42, 48, 54, and 60 hours after hepatectomy. In the second series of experiments, rats with and without HN2 were sacrificed 36 hours after the operation. In the third series, animals were killed 48 and 60 hours postoperatively. Control experiments on the effects of laparotomy, injection of saline after partial hepatectomy, and of treatment with HN2 in normal animals without hepatectomy were carried out.

The rats were weighed prior to operation as well as prior to sacrifice. The liver lobes removed at operation were weighed and promptly placed in a deep-freeze unit at about −20° C. These samples provided the normal, prehepatectomy control values. The regenerating liver tissue was carefully dissected out, weighed, and frozen in the same way. Random samples of normal and regenerating liver with and without HN2 treatment were taken for histological examination.

All specimens were allowed to thaw at room temperature. Representative portions of each liver were homogenized in 0.77 M sucrose in 0.01 M phosphate buffer at pH 7.6 in a Potter all-glass homogenizer. The final homogenates contained approximately 150 mg of fresh tissue/ml. Duplicate aliquots of each homogenate were used for direct enumeration of nuclei following suitable dilution in 3 per cent acetic acid containing 0.02 per cent methyl green, and for nucleic acid analyses with diphenylamine and orcinol following extraction by the method of Schneider (38). For purposes of calculation of nucleic acids per cell, each nucleus was assumed to represent one cell. The procedures outlined by Price and Laird (37) were closely followed in most respects.

RESULTS

Assessment of hepatectomy.—According to Higgins and Anderson (27) and Brues and Marble (6), 67–75 per cent of the liver was removed in successful partial hepatectomy by their technic. These authors also demonstrated that the average weight of the liver in rats weighing between 125 and 225 gm. is 3.8 per cent of body weight. In the present experiments, the average weight of the liver in eight animals which died during the operation was 3.7 per cent of body weight. The amount of liver removed in 23 animals, taken at random, was calculated to be 68 per cent of the average total liver weight (range 53–86 per cent). It was concluded that our operative procedure was comparable to that of these authors.

Assessment of treatment with HN2.—The most suitable dosage level for subcutaneous administration was chosen on the basis of the work of Anslow *et al.* (2), Hunt and Philips (31), and Graef *et al.* (24), who established a dose of 0.9–1.2 mg/kg as the LD_{50} for administration through the tail vein. It was determined that the concentration of HN2 required for the production of reliable decreases in the white cell count was slightly larger when given by subcutaneous injection. A single dose of 1.9 mg/kg at the time of hepatectomy was found to be accompanied in the first 24 hours by decreases in the white cell count to about 3,000 cells/cmm, but not to lower values; only a few of the rats died during this period. When animals were maintained for 48 and 60 hours, the white cell count frequently dropped below 3,000, and the number of fatalities was greater. The white cell count in untreated rats ranged between 5,000 and 12,000, with an average of 10,000.

In a number of animals, the weight of the spleen was also determined at autopsy to obtain further evidence on the systemic action of HN2. The spleen of untreated animals ranged in weight from 0.35 to 0.76 gm.; in animals treated with HN2, the weight of the spleen ranged from 0.10 to 0.53 gm.

Effect of partial hepatectomy without HN2 treatment.—Table 1 summarizes the changes in cellularity and in the levels of DNA and RNA per cell in regenerating liver. Comparison with the corresponding data of Price and Laird (37) shows excellent general agreement between the results of the two investigations. However, in the present series of experiments the accumulation of DNA did not reach so high a level as in the study by these authors, who reported a rise to 150–180 per cent of control levels at 24 hours after hepatectomy in three separate experiments.

TABLE 1

CELLULARITY AND NUCLEIC ACID LEVELS PER CELL IN REGENERATING RAT LIVER IN THE ABSENCE OF NITROGEN MUSTARD

Hours after hepatectomy	No. of animals	Cell count (Nuclei/mg wet wt)	DNA ($\mu\mu$g/cell)	RNA ($\mu\mu$g/cell)
0	106	190,000±2,700*	10.4±0.2*	30.6±0.7*
12	2	161,000	10.6	31.4
15	2	168,000	12.3	36.6
24	6	139,000±5,100	14.0±1.0	44.2±3.2
36	25	131,000±5,100	14.2±0.6	51.1±2.1
42	4	132,000	13.5	43.7
48	9	146,000±6,400	13.2±0.7	44.3±2.3
60	6	164,000±5,300	12.6±0.2	37.9±1.5

* These values are the standard deviations of the mean $= \frac{\sigma}{\sqrt{n}}$, where σ is the standard deviation of the observations in the sample and n is the number of observations.

Effect of HN2 on the accumulation of nucleic acids at 36 hours after hepatectomy.—Table 2 summarizes the data obtained in these experiments. The administration of a single dose of HN2 at hepatectomy not only does not interfere with the usual increases in nucleic acid concentration but actually brings about statistically significant increases in the accumulation of both DNA and RNA and a further decrease in the cellularity of the liver 36 hours after the operation.

This conclusion is supported by calculations on the basis of the entire organ. The average weight of the liver 36 hours after hepatectomy was 2.8 gm. in a representative number of control animals and 3.0 gm. in a similar number of treated animals. The average total content of nucleic acids in these livers was slightly higher in the HN2-treated group than in the untreated group (DNA, 5.8 and 5.4 mg/liver, respectively; RNA, 25 and 21 mg/liver, respectively).

The average prehepatectomy values for the cell count in the livers of treated and untreated animals were essentially the same; the average values for DNA as well as for RNA were identical in the two groups. All these values were in close agreement with the average values obtained in the entire series of 106 prehepatectomy livers which constituted the zero-time controls shown in Table 1.

This series of sixteen control rats and 31 rats treated with HN2 was obtained in three separate experiments with four to six control and eight to twelve treated animals each, performed at approximately monthly intervals. The most pronounced differences in nucleic acid levels between treated and untreated animals were found in the first experiment. The same trend was seen in the other two experiments, though the differences between treated and untreated animals were not so great. In view of the appreciable variation from animal to animal, which will be discussed below, the results obtained in this biological system with small groups of animals are somewhat suspect, and, therefore, statistical treatment of the results was possible only when all three experiments were pooled.

There is a significantly greater accumulation of RNA than of DNA in both treated and untreated animals at 36 hours. This difference in extent of accumulation between the two nucleic acids cannot be explained at the present time.[2]

TABLE 2

EFFECT OF HN2 TREATMENT ON CELLULARITY AND NUCLEIC ACID LEVELS PER CELL OF REGENERATING LIVER 36 HOURS AFTER HEPATECTOMY

Group	Control		HN2-treated
No. rats	16		31
Cell count (nuclei/mg wet weight)			
Prehepatectomy	203,000		198,000
Posthepatectomy	142,000		125,000
D/S_D* control vs. treated		2.4	
DNA ($\mu\mu$g/cell)			
Prehepatectomy	9.8		9.8
Posthepatectomy	12.4		14.8
D/S_D control vs. treated		3.0	
RNA ($\mu\mu$g/cell)			
Prehepatectomy	30.2		30.2
Posthepatectomy	50.7		62.5
D/S_D control vs. treated		3.2	

$$* \; D/S_D = \frac{M_1 - M_2}{\sqrt{\sigma^2_{M_1} + \sigma^2_{M_2}}}.$$

Effect of HN2 on the accumulation of nucleic acids at 48 and 60 hours after hepatectomy.—Pre-

[2] The possibility was considered that some of the "orcinol color" in these experiments should be attributed to glycogen rather than to RNA. Preliminary experiments indicated, however, that the addition of glycogen, at concentrations higher than those to be expected in regenerating rat liver, to homogenates prior to the Schneider procedure did not affect the diphenylamine reaction for DNA and brought about only a small additional "orcinol color." The magnitude of this possible glycogen blank was not sufficient to account for the significantly greater accumulation of RNA.

liminary results obtained at these time periods following hepatectomy and administration of the single dose of HN2 are summarized in Table 3. It would appear that treatment with the drug has no demonstrable effect on the DNA or RNA content of the cells of regenerating liver at these times. The data of Friedenwald *et al.* (19) on corneal epithelium and of Landing and associates (34) on regenerating liver indicate that inhibition of mitosis after a single dose of nitrogen mustard may be expected to disappear in 36–48 hours.

Control experiments.—It was ascertained in preliminary experiments that there were no differences in the analytical results between portions of the same liver assayed immediately after removal from the animal and after maintenance at −20° C. for variable periods of time.

In agreement with Price and Laird (37), it was found that the DNA and RNA values in livers removed 36 hours after laparotomy without hepatectomy showed no appreciable variations from the control values.

The administration of the usual dose of HN2 to rats after laparotomy or to rats not subjected to any surgical manipulation had the expected effect on the white cell count but brought about no changes in the DNA or RNA levels per cell in livers removed 36 hours after the injection. Physiological saline, when administered to normal, sham-operated, or partially hepatectomized animals, had no effect on the white cell count or nucleic acid values.

Prior to the analysis of liver homogenates from treated animals, it was desired to determine the effect of HN2 on the diphenylamine and orcinol reactions employed in the nucleic acid estimations. For this experiment, 10 mg. HN2 was dissolved in 10 ml. phosphate buffer, and the solution was allowed to stand for ½ hour at body temperature to permit cyclization to occur. This solution was diluted with 5 per cent trichloroacetic acid, and aliquots were added to portions of the final 5 per cent trichloroacetic acid extracts prepared from homogenates of normal rat liver. The level of HN2 in the final extract was about 10 times that which would be expected to be present in extracts prepared from livers removed from the animals shortly after the injection of HN2. These analyses were compared to analyses of the same extracts without added HN2. There was no appreciable and reproducible difference between these results.

Histological findings.—Since all the results of the present investigation were obtained by a single procedure, i.e., homogenization of the liver and direct enumeration of nuclei in an aliquot of the homogenate, it was of importance to confirm the significant differences in cellularity before and after hepatectomy by an independent method. For this purpose, representative samples of several livers were fixed in Bouin's solution, and histological slides stained with hematoxylin-eosin were prepared under identical conditions. The number of nuclei in random areas of these slides was determined with a micrometer eyepiece, and the values after hepatectomy with or without HN2 treatment were related to the prehepatectomy values. The two methods yielded essentially the same picture of the comparative cellularity of these livers, within the limits of error of the two procedures.

Qualitative examination of these slides revealed easily detectable differences which are demonstrated in Figures 1–3. The stroma of the regenerating liver is less compact than the control. The cells are larger in the former, and the nuclei are also consistently larger, although they show significantly greater variation in size. The frequency of mitotic figures is high in the regenerating liver sections and virtually absent in the control liver. These findings are in general agreement with the data of Price and Laird (37) and Stowell (43). A comparison of the histological appearance of regenerating liver from nitrogen mustard-treated and untreated animals fails to demonstrate a marked difference. It is our impression that the frequency of mitotic patterns is lower in the former than in the latter.

TABLE 3

EFFECT OF HN2 TREATMENT ON CELLULARITY AND NUCLEIC ACID LEVELS PER CELL OF REGENERATING LIVER 48 AND 60 HOURS AFTER HEPATECTOMY

GROUP	48 HOURS Control	48 HOURS HN2-treated	60 HOURS Control	60 HOURS HN2-treated
No. rats	4	5	4	5
Cell count (nuclei/mg wet wt)				
Prehepatectomy	174,000	172,000	208,000	210,000
Posthepatectomy	157,000	144,000	164,000	188,000
DNA ($\mu\mu$g/cell)				
Prehepatectomy	8.6	10.5	9.6	8.9
Posthepatectomy	12.4	13.6	12.7	12.6
RNA ($\mu\mu$g/cell)				
Prehepatectomy	31.4	39.4	28.4	26.6
Posthepatectomy	47.4	58.0	39.3	43.4

DISCUSSION

Before attempting to interpret the results of the present investigation in terms of the mechanism of action of nitrogen mustard, it is important to evaluate the method by which the results were obtained and the suitability of the biological system which has been employed.

Evaluation of the method.—Several procedures based on entirely different approaches have been

used for the estimation of the nucleic acid content of various mammalian cell populations. The simplest and least time-consuming method consists of homogenization of a sample, direct enumeration of nuclei in one aliquot of the homogenate, and determination of the nucleic acid content in another aliquot. This approach has been subjected to an extensive investigation in a wide variety of tissues during the last 2 years and has been shown to yield valid and reliable results.[1]

In the present series of experiments, the following findings have confirmed the conclusion that this simple method is a trustworthy guide: (*a*) The average value obtained for the DNA content of normal rat liver in 106 animals was 10.4 ± 0.2 $\mu\mu g$/cell (cf. Table 1); this value is in excellent agreement with the values obtained by a variety of other procedures (11, 13, 14, 17, 37), though higher than the value reported by Cunningham and collaborators (12). (*b*) The accumulation of nucleic acids after hepatectomy in these experiments follows the pattern described by Price and Laird (37) in most respects; the careful evaluation these authors made of the suitability of this method for studies on regenerating liver offers further support for the reliability of the present data. (*c*) It is realized that if the characteristic decrease in cell count observed in the early periods after hepatectomy were an artifact produced by a supposable fragmentation of nuclei during homogenization, then the increases in nucleic acid concentrations calculated on a cell basis would be entirely illusory. This decrease in cell count was, however, confirmed by the histological evidence which has been alluded to.

Evaluation of the biological system.—Regenerating rat liver offers advantages as well as disadvantages for the investigation of the biochemical mode of action of antitumor agents. This model system of non-neoplastic growth is characterized in its early stages by a mitotic index comparable to that of anaplastic transplanted tumors (6, 44). Moreover, liver regeneration is an unfailing consequence of adequate partial hepatectomy, so that an ample supply of experimental material can be produced at will.

The chief drawback of this system is that, although regeneration never fails to follow hepatectomy, the rate of regeneration, at least from the point of view of nucleic acid synthesis, is subject to considerable variation. This variation may be caused by a number of factors (7, 8, 15, 30 [pp. 82]), among them, the nutritional status of the animals before and after operation, diurnal and seasonal variations in mitotic activity, environmental temperature, strain, age, and weight of the animals, severity of the trauma produced by the operation and concomitant anesthesia, intensity of the humoral stimulation of regeneration, etc. While attempts were made to control these factors in the present experiments, it is recognized that some of them must have continued to operate at least to some extent.

In many experimental situations, variations of this magnitude might not have any crucial significance. The following considerations, however, indicate that they become an important factor in experiments with this tissue.

One of the biochemical processes which must take place in most biological systems before a cell can divide is an increase in its nucleic acid content. A variety of findings (cf. 30 [pp. 101–3]) suggest that the DNA content reaches approximately twice the resting cell level shortly before the actual division of the cell into two daughter cells. In regenerating liver, the cells of the remaining portion of liver following hepatectomy may then be envisaged to prepare for their first mitosis by doubling their DNA content. If all the cells were carrying out this function at exactly the same time, it would be theoretically possible to select a moment after hepatectomy at which the DNA content of the "average cell" would be twice that of the prehepatectomy sample. Such a finding is, of course, highly unlikely in view of the differences in operative trauma, humoral stimulation of regeneration, access to building blocks, synthetic ability, etc., which must exist between cells in different areas of the remaining lobe of the liver. At any one time, some cells will be in the resting stage, others will be carrying on nucleic acid synthesis prior to division, others will be just ready to divide, yet others will have newly arisen by mitosis. The DNA content of the "average cell" at this time will then be some value intermediate between normal and twice normal, and the quantitative range of expression of any effect of a chemotherapeutic agent on this value will be severely limited. Therefore, relatively large numbers of animals must be used in the control and treated groups to permit a statistical evaluation of any change which may be observed, and the significance of the result is not necessarily commensurate with the effort which is required to document it. This practical drawback must be taken into account when the suitability of regenerating rat liver for these studies is assessed.

Effect of HN2 on the nucleic acid content of the "average cell."—Two conclusions may be drawn from the data which have been presented. First, the administration of nitrogen mustard does not prevent the accumulation of nucleic acids which

normally takes place in the early periods of liver regeneration. The present experiments do not provide any information concerning the rate of this accumulation, but it is clear that at 36 hours after hepatectomy the treated cells do not contain less nucleic acid than the untreated cells. Second, the present data actually demonstrate that there is an appreciably greater accumulation of nucleic acids in the treated cells than in the controls. The significance of this finding may be discussed briefly.

In the simplest interpretation, the nucleic acid concentration per cell at any time after hepatectomy may indicate the number of liver cells which have not yet divided for the first time; the higher this value in relation to the concentration before hepatectomy, the larger the number of cells yet waiting to divide. The significantly higher levels of nucleic acids in the "average cell" after nitrogen mustard treatment would then indicate that at 36 hours posthepatectomy a significantly greater number of liver cells have not yet entered the first mitosis. The absence of any demonstrable difference in nucleic acid levels in treated and untreated cells at 48 and 60 hours suggests that cell division is postponed for only a relatively small number of hours when a single dose of the drug is given.

The conclusion that this postponement of mitosis in a significant number of cells is accompanied by a net rise in the nucleic acid content of the "average cell," i.e., that the cells in which mitosis is blocked already appear to contain the high premitotic level of nucleic acid, is the central result of this investigation. This study cannot provide any direct evidence for or against an inhibition of synthesis of nucleic acid by HN2; it does, however, demonstrate that inhibition of cell division by this agent in regenerating liver is not mediated primarily through such an inhibition of this synthetic process. It may be postulated that HN2 interferes with mitosis either (*a*) by a mechanism not directly involving the nucleic acids or (*b*) by blocking nucleic acid utilization; the decreased incorporation of precursor into nucleic acid purines observed by Skipper *et al.* (40), Goldthwait (23), and others might then be a secondary consequence of the fact that further synthesis would cease once the doubling of nucleic acids in these blocked cells has taken place. The data which have been presented would also be consistent with the assumption that both synthesis and utilization of nucleic acids were interfered with by this drug, but that the inhibition of utilization was significantly more pronounced than the inhibition of synthesis.

It should be stressed that an attempt to correlate incorporation data with formate-C^{14} obtained in the viscera of mice and static nucleic acid analyses in regenerating rat liver is hazardous at best and that generalizations from any particular system to a variety of tissues would not appear permissible at the present time. Therefore, experiments are now in progress to assess the incorporation of nucleic acid precursors in regenerating liver and the effect of other drugs on the nucleic acid levels in this and other biological systems.

SUMMARY

1. The suitability of regenerating rat liver for studies on the biochemical mechanism of action of cancer chemotherapeutic agents has been evaluated.

2. In agreement with data obtained by other investigators, it was found that there was an accumulation of nucleic acids in the liver cells in the early periods of regeneration following partial hepatectomy. This accumulation reached its peak 36 hours after hepatectomy.

3. The subcutaneous injection of a single dose of nitrogen mustard was followed by a significantly greater accumulation of nucleic acids in the liver cells at 36 hours after hepatectomy. At 48 and 60 hours, there was no longer a demonstrable difference in the nucleic acid levels per cell between treated and untreated animals.

4. It appears that in this biological system the inhibition of mitosis by nitrogen mustard was not mediated primarily through an inhibition of nucleic acid synthesis, as suggested by the work of other investigators with isotopically labeled precursors of nucleic acids.

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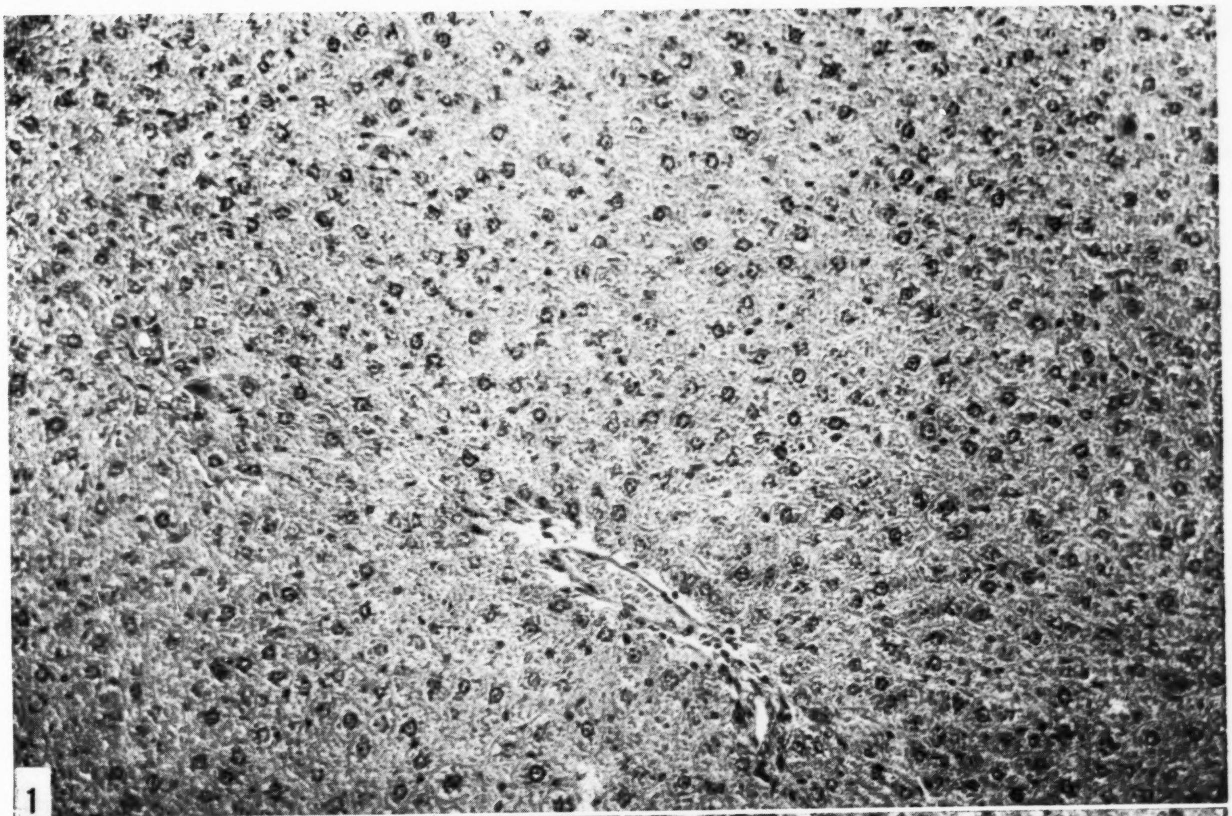
FIG. 1—Normal rat liver. ×150.

FIG. 2.—Regenerating rat liver 36 hours after hepatectomy. ×150.

FIG. 3.—Regenerating rat liver 36 hours after hepatectomy and after a single injection of HN2. ×150.



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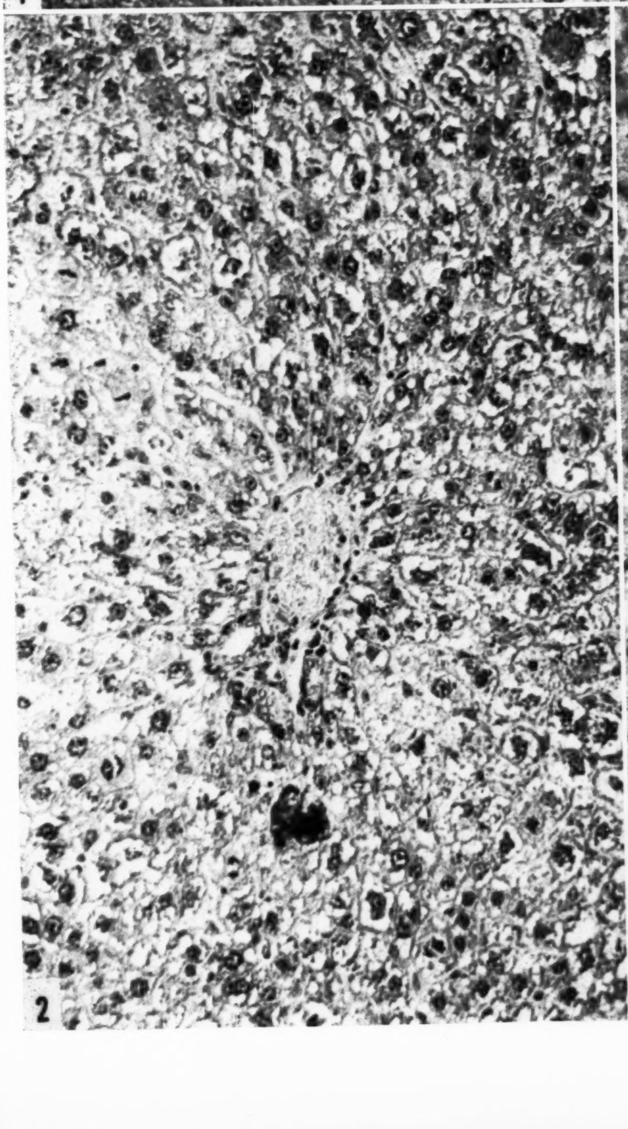

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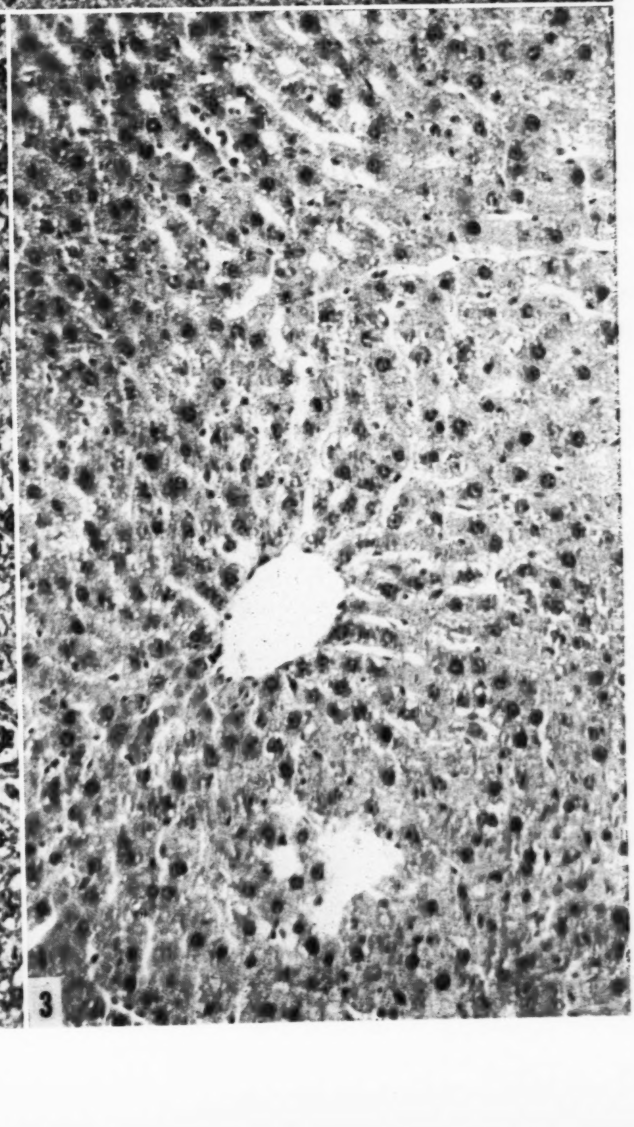

3

Metabolism of Neoplastic Tissue

III. Diphosphopyridine Nucleotide Requirements for Oxidations by Mitochondria of Neoplastic and Non-neoplastic Tissues*

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In previous publications we reported that oxidative processes of the citric acid cycle in tumor homogenates (29) or mitochondria isolated therefrom (30) are limited by the loss of diphosphopyridine nucleotide activity and that oxygen consumption can be restored by the addition of this coenzyme to the medium in relatively high concentration. The present report represents a further study of the effect of DPN on the oxidative behavior of tumor mitochondria, together with a comparison with mitochondria of various non-neoplastic tissues in regard to oxidative activity toward pyruvate and components of the citric acid cycle.

METHODS

Transplanted tumors were maintained by subcutaneous implantation in adult mice of the C3H, Swiss, or A strain. After reaching a diameter of approximately 1 cm., they were dissected from the decapitated, exsanguinated animals, and mitochondria from these tissues were prepared exactly according to the procedure of Schneider (22). The washed mitochondria were finally suspended in cold, isotonic sucrose unless otherwise specified. All operations were carried out at $<5°$ C. The basic medium consisted of the following substances in the designated final concentrations: $MgSO_4$, 3×10^{-3} M; sodium fumarate ("sparker") 7×10^{-5} M; ATP, 2×10^{-3} M; cytochrome c, 4×10^{-5} M; phosphate buffer (pH 7.4), 6×10^{-3} M; KCl, 6.7×10^{-2} M; DPN, 1.9×10^{-3} M. To this there was added substrate (0.006 M) and 0.4 ml. of the mitochondrial suspension, representing 200 mg. of the original tumor tissue or 133 mg. of original normal tissue, to a final volume of 1.6 cc. in chilled Warburg vessels. The flasks were shaken at 38° C., with air as the gas phase. Oxygen uptake measurements were begun after an initial 7 minutes of equilibration.

Substrates and other material.—Citrate, α-ketoglutarate, succinate, malate, fumarate, pyruvate (all added as the sodium salts), cytochrome c, adenosine triphosphate (ATP), and diphosphopyridine nucleotide (DPN) were commercial products. The DPN was obtained either from Sigma Chemical Co. or the Pabst Laboratories, and assayed from 40 per cent to 90 per cent pure by the enzymatic procedure of Racker (21). Reduced DPN was prepared by the procedure of Ohlmeyer (17), and the contaminating sulfite and sulfate were removed according to Lehninger's modifications (11). This material was found to be 75 per cent pure by measurement of the absorption at 340 mμ with use of the molar extinction coefficient, 6.22×10^6 sq cm/mole (7). Triphosphopyridine nucleotide, assaying 40 per cent pure by the Zwischenferment assay (28), was prepared by the method of LePage and Mueller (14). Nitrogen determinations on the tissues were made by the micro-Kjeldahl procedure of Ma and Zuazaga (15).

RESULTS

Pyruvate oxidation by hepatoma-cell components.—Previous investigations have demonstrated that

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the enzymatic equipment for oxidation of fatty acids and citric acid cycle components in normal tissues is restricted to the cytoplasmic granules known as mitochondria (5, 6, 9, 23, 25). Results of a study of the distribution of pyruvic oxidation activity among the centrifugally separated cell components, as shown in Table 1, demonstrate that be assumed that the low activities observed in the nuclear, microsomal, and supernatant fractions are due to contamination by mitochondria. The whole homogenate displays an appreciable endogenous oxygen uptake, which is greatly augmented by addition of DPN, with or without substrate. This activation of the oxidation of endogenous

TABLE 1

PYRUVATE OXIDATION IN CELLULAR CONSTITUENTS OF MOUSE HEPATOMA 98/15

			Oxygen consumption					
			O_2, μl/100 mg tissue			O_2, μl/mg N		
Tissue fractions*	Total N†	Percent of whole homog.	None	Additions DPN	DPN+pyruvate	None	Additions DPN	DPN+pyruvate
Homogenate	2.08	100	46	195	164	19.8	84	72
N_{W_2}	0.508	24.4	2.6	14	14	5.1	28	28
M_{W_2}	0.275	13.2	2.3	14	58	8.4	52	211
P_W	0.222	10.7	1.6	4.5	2.1	7.2	20	9.5
S_2	1.150	55.3	5.8	41	31	5.0	36	27

* Notation is that of Schneider (21).

† Mg nitrogen per 100 mg. fresh tissue.

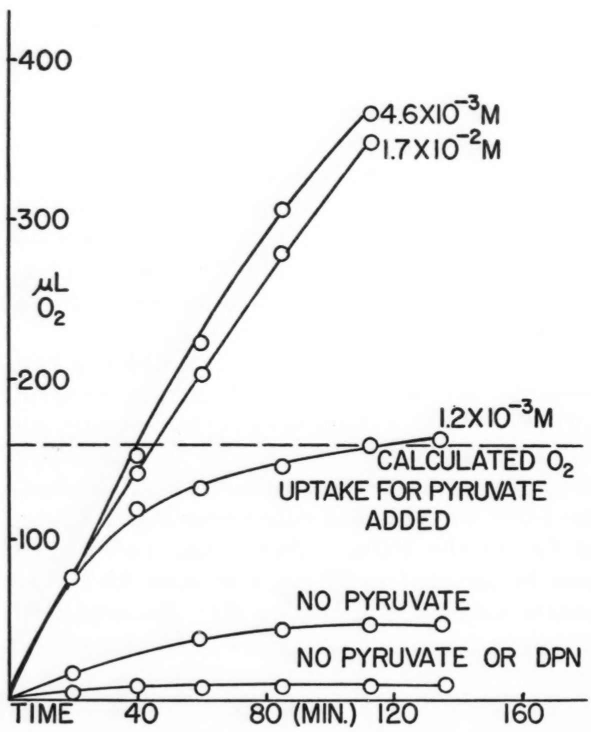

CHART 1.—Effect of substrate concentration on the oxidation of pyruvate by mouse hepatoma mitochondria. Ordinate represents the μl. of oxygen consumed by mitochondria equivalent to 200 mg. of original tissue (wet wt.).

the hepatoma 98/15 also has essentially all this activity localized in the mitochondrial fraction. In agreement with previous fractionation data published by Schneider (23), the mitochondrial fraction contained about 13 per cent of the total nitrogen, and this was the only one of the fractions showing high oxidative activity toward pyruvic acid. Since the separations are not absolute, it can

substrates by DPN has been found to be characteristic of whole homogenates of a variety of tumors. The sum of the oxygen uptakes of the separate fractions is approximately two-thirds that of the whole homogenate, indicating that considerable inactivation occurs during separation of the fractions.

Effect of pyruvate concentration.—The characteristic oxidative behavior of DPN-fortified mitochondria of neoplastic tissue is shown in Chart 1, in which pyruvate oxidation was studied in particles obtained from mouse hepatoma 98/15. At high substrate concentrations oxygen consumption proceeded at linear rates for approximately 2 hours and was observed to be maximal at 0.005 M. It seems highly probable that oxidation of pyruvate goes to completion in this system, since the oxygen uptake at 0.001 M pyruvate leveled off at a value close to that calculated for complete oxidation of the substrate. Addition of DPN consistently caused an appreciably increased oxygen consumption in the absence of substrate, which is probably due to stimulation of the oxidation of the "priming" agent or of endogenous carbon. This pattern of oxidative activity was displayed for citric acid cycle components as well as pyruvate, and by mitochondria from non-neoplastic as well as neoplastic tissue.

Specificity of DPN activation.—In the absence of DPN, pyruvate oxidation in mitochondria of hepatoma and rhabdomyosarcoma was not activated by addition of TPN, flavin adenine nucleotide, coenzyme A, or any of the adenine nucleotides. Addition of fluoride, which Potter and Lyle (19) found to stimulate respiration of the Flexner-Jobling tumor, had no effect on oxygen consump-

tion, either with or without DPN, when tested with our tumors. The only substance having a similar effect was reduced DPN (Table 2). It was expected that differences in activation might have been observed as the result of differences in permeability of the mitochondrial membrane for the two forms of the coenzyme. However, as shown in Table 2, activation was the same at comparable concentrations. In accord with expectations, hepatoma mitochondria readily oxidized reduced DPN; as shown in Table 2, the oxygen uptake corresponded closely to the theoretical amount required for oxidation to DPN.

TABLE 2

REPLACEMENT OF DPN REQUIREMENT BY DPN-H_2 IN PYRUVATE OXIDATION BY 98/15 HEPATOMA MITOCHONDRIA

Additions DPN (2×10^{-3} M)	DPN-H_2 (2×10^{-3} M)	Pyruvate (0.01 M)	O_2 uptake (μl/mg N/30 min)
−	−	−	18.7
+	−	−	55
−	+	−	30
−	−	+	46
−	+	+	146
+	−	+	146
−	+*	−	200

* In this experiment DPN-H_2 equivalent to 17.3 μM of the pure material was incubated for 3 hours at 38° C. with mitochondria representing 200 mg. of original tissue. The observed oxygen uptake compared favorably with the theoretical oxygen uptake of 193 μl.

Relationship between oxidative activity and mitochondrial content.—Since the reactions of the citric acid cycle seemed to be localized in the mitochondria, it appeared probable that many of the quantitative differences in oxidative activity between different tissues could be referable to variability in mitochondrial content. Evidence for the correctness of this postulate has already been reported (20, 23, 30), and these observations have been extended in the present report. Data on pyruvate oxidation and mitochondrial nitrogen content for a number of neoplastic and non-neoplastic tissues are shown diagrammatically in Chart 2. A marked difference in mitochondrial content[1] between the two types of tissue is immediately evident; indeed, it was surprising to observe the extreme variability displayed by the various tissues. In view of the generally low mitochondrial content of tumor tissues, one can readily understand why such tissues have been reported as deficient in such mitochondria-bound factors as succinic dehydrogenase and cytochrome c (4, 24).

[1] The validity of basing mitochondrial content on the nitrogen content without knowledge of their composition may be questioned; however, in the absence of other easy methods, this may be regarded as a fair approximation for the purpose intended.

A rather good correlation is displayed in Chart 2 between the mitochondrial content and the activity toward pyruvate oxidation, emphasizing again the quantitative as well as qualitative similarity of mitochondria of different tissues in their capacity for oxidation of pyruvate.

Oxidation of citric acid cycle components.—These studies have been further extended to include comparisons between normal and neoplastic mitochondria in the oxidation of citric acid cycle components and the effect thereon of the addition of DPN. The results of this survey are collected in Table 3. To aid in these comparisons, all oxygen uptake values are on a per milligram nitrogen basis. In agreement with the data of other investigators (3, 9), all the substrates were readily oxidized by mitochondria of liver and kidney, and little or no enhancement occurred with addition of DPN. In those instances in which DPN increased the oxygen uptake, the effect was due to better maintenance of the oxygen consumption rather than to an increase of the initial rate. In contrast

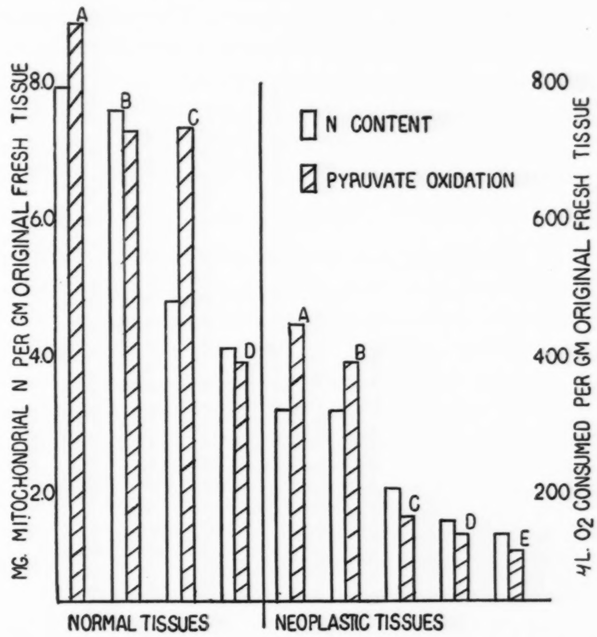

CHART 2.—Pyruvate oxidation versus nitrogen content of mitochondria of normal and neoplastic tissues. The unshaded bars on the left of each set of columns represent the mg. mitochondrial nitrogen per gram of original whole tissue. The shaded bars on the right side of each set of columns represent the net respiratory activity in μl. of O_2 consumed per gram fresh tissue per 30 minutes. Conditions of assay are outlined in the experimental section. Each column represents an average of two or three determinations. Normal tissues: column *A*, rat liver; *B*, mouse liver; *C*, mouse kidney; *D*, rat brain. Neoplastic tissues: column *A*, mouse hepatoma (A strain); *B*, mouse hepatoma (C3H strain); *C*, sarcoma 37; *D*, rhabdomyosarcoma; *E*, rat hepatoma.

with liver and kidney, oxygen uptake was relatively low in the unfortified brain mitochondria in the presence of all substrates except succinate but was enhanced considerably with DPN addition.[2] In this respect, brain mitochondria resemble those of the neoplastic tissues. All four tumors listed in Table 3 exhibited essentially the same oxidative pattern. In the absence of added DPN, oxygen uptake was invariably and consistently high; the only substrate whose oxidation was not increased by DPN addition was succinate. The activation of citrate oxidation by DPN was somewhat surprising, since the first dehydrogenase to act on this substance is isocitric dehydrogenase, which is generally considered to be specific for TPN (16). It is recognized, of course, that DPN would be required for the further oxidation of the products of isocitrate oxidation through the citric acid cycle, hence DPN should be expected to enhance the over-all oxidation of citrate. On the other hand, oxidation of citrate to ketoglutarate should proceed unimpeded in the absence of DPN, or should require TPN. Since this does not occur, we can only conclude either that DPN can function as electron carrier for isocitric dehydrogenase in mitochondria or that DPN is exerting some activating effect other than in electron transport.

DISCUSSION

It has been known for many years that slices of tumor tissue exhibit respiratory activities, which, though not generally so high as those of such highly metabolically active tissues as kidney, heart, and liver, are of similar magnitude to these and are within the range of any representative group of non-neoplastic tissues (2, 27). Isotopic tracer studies have also demonstrated the ability of tumor tissues to carry out the oxidation of fatty acids, glucose, and their intermediates (18, 29) at rates comparable to a variety of non-neoplastic slices. Enzyme assays have shown that the individual activities of the citric acid cycle occur in tumor tissues with the same order of magnitude as in non-neoplastic tissues (31). The present findings supplement these studies by establishing the ability of mitochondria of neoplastic tissues to carry out the complete oxidation of pyruvate and citric acid cycle components. The approximately equal activities of "neoplastic" and "non-neoplastic" mitochondria on a per milligram nitrogen basis emphasize that mitochondria from various cells are fundamentally similar in oxidative activities and indicate also that at least some of the reported differences in oxidative enzymes are due to variations in mitochondrial content rather than to differences in the enzymatic equipment of the mitochondria. That tumor mitochondria are able to carry out citric acid cycle oxidations has recently been shown also by other investigators. Potter and Lyle (19) reported that whole homogenates of the Flexner-Jobling carcinoma can oxidize a mixture of pyruvate and fumarate when fluoride is added to depress organic phosphate breakdown; and they attributed the previous failure of such systems to oxidize oxalacetate to an inability of phosphorylating processes to keep pace with the rapid dephosphorylation of ATP. More recently, Siekevitz, Simonson, and Potter (26) reported that different tumor homogenates display different rates of phosphate "turnover" and that good oxidation can be observed in tumor homogenates when these opposing activities are balanced by the addition either of dinitrophenol or fluoride. In a study of oxidative phosphorylation, Kielley (10) found that phosphorylative oxidation of succinate, glutamate, and α-ketoglutarate occurred in a transplantable mouse hepatoma; and, in agreement

TABLE 3

DPN REQUIREMENT FOR OXIDATION OF KREBS CYCLE COMPONENTS

Values are in oxygen consumed per mg. mitochondrial nitrogen per 30 minutes

	NORMAL TISSUE MITOCHONDRIA								TUMOR MITOCHONDRIA							
	Mouse liver		Rat liver		Mouse kidney		Rat brain		Hepatoma 7A77		Hepatoma 98/15		Sarcoma 37		Rhabdomyosarcoma	
SUBSTRATE	−DPN	+DPN	−DPN	+DPN	−DPN	+DPN	−DPN	+DPN	−DPN	+DPN	−DPN	+DPN	−DPN	+DPN	−DPN	+DPN
None	37	49	43	45	53	91	40	50	15	44	19	36	28	45	16	36
Pyruvate	150	145	139	154	279	249	38	146	30	250	17	196	51	150	21	105
Citrate	176	215	146	153	299	296	22	116	14	153		153		68	4	137
α-Ketoglutarate	142	180	118	154	212	335	43	116	100	214		119		99	85	172
Succinate	145	165	147	128	230	284	114	104	125	116	82	79	170	204	170	118
Fumarate	130	156	106	154	95	220	35	101	15	143	48	98				
Malate			160	164			69	127	33	176		82		136	56	99

[2] Recently, Brody and Bain (1) reported specific oxidative activities of brain mitochondria similar to those reported here, although DPN does not appear to be required for maximal activity in their system. This discrepancy can probably be attributed to the differences in procedure; they used higher centrifugation speeds, more washings, and suspended the mitochondrial fraction in distilled water prior to introduction into the Warburg vessels.

with our findings, observed a more definite requirement of DPN in hepatoma than in liver mitochondria for the two latter substrates whose oxidation requires this co-factor.

In our hands, the DPN requirement for oxygen uptake was the most consistent and characteristic feature of the tumor mitochondria. It was observed in every experiment with all tumors, whether rapid or slowly growing, and with DPN from commercial sources, as well as with material made in our laboratory. We recognize, however, that the methods of preparation of mitochondria are largely empirical; hence, in view of the many obvious differences in experimental conditions among different investigators, such as the tonicity of media, speed of centrifugation, concentrations of substrates and co-factors, temperature of incubation, etc., it is not too surprising that Potter *et al.* (26) found that certain tumor homogenates consume oxygen without DPN addition or that Kielley observed a pronounced DPN requirement only after short aging periods. Since a DPN requirement can be created for liver and kidney mitochondria by such mild treatments as freezing and thawing, hypotonicity, or aging at 0° (8), it seems probable that the DPN requirement of tumor mitochondria does not represent a fundamental difference in the metabolism of neoplastic tissue but is due rather to structural characteristics of the mitochondria which are manifested by a loosening of the binding of DPN.

The reasonable assumption that mitochondria of different cell types may vary in their ability to bind DPN or other coenzymes may serve to explain certain observed differences in metabolism among various tissues. For example, a weakness in the binding of DPN by tumor mitochondria, such as is suggested by the present study, may provide a simple, rational explanation for one of the most perplexing features of the metabolism of tumor cells—namely, their high aerobic and anaerobic glycolysis. The extensive studies of LePage (12, 13) have clearly established the fact that whole tissue homogenates, when fortified with DPN and other co-factors, can carry out glycolysis of hexose diphosphate at rates which are far higher than those observed for tissue slices acting on glucose, and have further shown that similar glycolytic levels are displayed by such fortified homogenates of both neoplastic and non-neoplastic tissues. These findings suggest that the potential glycolytic capacities of these cells are similar, but that the rates of these processes may be controlled and regulated in the intact cell by the intracellular distribution of these co-factors. Inasmuch as the glycolytic enzymes are in the soluble portion of the cytoplasm, it would be expected that glycolysis would be higher in tumor cells, in which DPN is loosely bound, and, hence, would be in correspondingly higher concentrations in the soluble portion of the cell where the process occurs. Conversely, glycolysis would be expected to be low in cells in which DPN is more strongly bound and, hence, not available for maximal activity of the electron-transferring steps of glycolysis. It is noteworthy in this connection that rat brain, whose mitochondria displayed a DPN requirement in this study, is one of the small number of non-neoplastic tissues which has a high rate of glycolysis (27). The correlation of glycolytic activity with the intracellular distribution of DPN is now under further investigation.

SUMMARY

The oxidation of pyruvate and citric acid cycle components occurs readily and consistently in mitochondria of neoplastic tissues when these are fortified by the addition of DPN in rather high concentration. This DPN requirement cannot be replaced by other known coenzymes or adenine nucleotides. On a per milligram nitrogen basis, oxidations by such fortified mitochondria of neoplastic tissues were quantitatively similar to their non-neoplastic counterparts. The suggestion is made that mitochondria of neoplastic tissues do not bind DPN as strongly as certain normal tissues, and that this may result in a correspondingly higher level of this coenzyme in the soluble portion of the cell cytoplasm. It is further suggested that this phenomenon may account for the charasteristically high glycolysis rates of intact tumor cells.

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Metabolism of Neoplastic Tissue. IV. A Study of Lipid Synthesis in Neoplastic Tissue Slices *in Vitro**

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Although considerable effort has been expended on the study of protein synthesis in neoplastic tissues (6–8), relatively little attention has been given to another synthetic process occurring in living cells, i.e., formation of the fatty acid carbon chain. The only data available at present with respect to this process in tumor cells are several experiments mentioned by Zamecnik *et al.* (8) and Olson (3) which indicate that the primary hepatoma of the rat, induced by administration of *p*-dimethylaminoazobenzene, can incorporate carbon of radioactive acetate and glucose into fatty acids *in vitro*. In the present study, the incorporation of acetate and glucose carbon into the lipids of slices of a series of transplanted tumors was measured, with methyl-labeled acetate and uniformly labeled biosynthetic glucose.

EXPERIMENTAL PROCEDURES

The tumors were maintained by subcutaneous implantation in rats of a special strain (1) or mice of the A and C3H strains. They were used when approximately 1 cm. in diameter.

The methyl-labeled sodium acetate was obtained from Tracerlab, Inc., and the labeled glucose from the Oak Ridge National Laboratory, both having been allocated by the Atomic Energy Commission. The acetate was used in 0.01 molar concentration after dilution with normal acetate to an activity of between 90,000 and 180,000 c.p.m., and the glucose was employed in a final molar concentration of 0.0044 after dilution to an activity of 32,300 c.p.m.

The experimental procedures were essentially the same as previously reported for a study of fatty acid synthesis in non-neoplastic tissues of the rat (2). Approximately 5 gm. of fresh tissue slices were shaken in modified Warburg vessels (5) for 4 hours at 38° C. in 40 ml. of a saline medium containing the labeled substrate. Oxygen was present in the gas phase, and respiratory carbon dioxide was collected in alkali in the center well. At the close of the incubation period, the flask contents were acidified by tipping in sulfuric acid from the sidearm, and, after allowing 10 minutes for complete absorption of the CO_2, the carbonate was assayed for radioactivity as described previously (2). The slices were extracted with alcohol and ether, and the fatty acids and nonsaponifiable matter were isolated according to standard procedures. In some experiments phospholipide fatty acids were separated from nonphospholipid fatty acids essentially as previously described (4). All the animals were fed ad libitum prior to use, but no special efforts were made to obtain optimal conditions for fatty acid synthesis, such as were employed in a previous study (2). The animals were fed a diet of Allied Mills Laboratory checkers.

Rates[1] of incorporation of the labeled carbon into the isolated components are reported as conversion capacities (C.C.), this value being defined as the micro-atoms of substrate carbon incorporated into the product per gram dry weight of tissue per hour; it is calculated from the following formula:

$$\text{C.C.} = \frac{\mu \text{ atoms carbon of product} \times \text{sp. act. of product}}{\text{gm. dry wt. tissue} \times \text{hours} \times \text{sp. act. of substrate}}.$$

To obtain specific activities, the various products were converted in some instances to barium carbonate by oxidation and assayed as such; in most cases, however, the isolated fatty acids and nonsaponifiable matter were assayed directly, and the values thus obtained were converted to their corresponding activities as barium carbonate by application of a factor obtained experimentally. All activities were measured as infinitely thick layers

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[1] Calculation of an hourly rate of lipogenesis from these experiments extending over 4 hours is justified on the grounds that this process has been observed to proceed linearly under these conditions in liver slices (2).

on a standard disk 7.5 sq. cm. in area and are reported in counts per minute per disk.

RESULTS

The ability of a variety of transplanted neoplasms of the rat and mouse to incorporate acetate carbons into fatty acids and nonsaponifiable matter is surveyed in Table 1. The results established

TABLE 1

INCORPORATION OF ACETATE INTO LIPIDS AND RESPIRATORY CO_2 OF SLICES OF NORMAL AND NEOPLASTIC TISSUES

Values are in conversion capacities (C.C.)

Tissue	No. of Exps.	Fatty acids	Nonsaponifiable lipid	Respiratory CO_2
Liver of normal rat	2	0.51, 1.99	0.72, 1.78	4.26, 20
Liver of hepatoma-bearing rat	3	0.39–2.47	0.23–1.39	25
Rat hepatoma	4	0.16–0.41	0.04–0.62	4.5
Liver of hepatoma-bearing mouse	2	0.26, 1.17	0.09, 0.17	12, 24
Mouse hepatoma (7A77)	4	0.90–1.75	0.28–0.63	8.3–11.1
Kidney of normal rat	3	0.94–1.95	0.30–0.43	88–134
Kidney of hepatoma-bearing rat	1	1.63	0.067	103
Normal rat spleen	1	0.38	0.025	12.4
Normal rat testis	1	0.04	0.02	9.9
Normal rat brain	1	0.03	0.009	1.14
Normal rat muscle	1	0.02	0.001	1.84
Mouse rhabdomyosarcoma	2	0.12, 0.11	0.11, 0.12	1.28
Mouse mammary tumor	1	1.30	0.11	8.6
Mouse sarcoma 37	2	0.07, 0.11	0.02, 0.02	0.71, 1.03

beyond doubt that neoplastic cells can carry out lipogenesis, although the rates are rather low compared to those of liver and kidney slices. Of the various neoplasms studied, the mouse hepatoma 7A77 displayed the highest rates of lipogenesis, approaching those of the non-neoplastic liver cells; in general, however, the other tumors resembled nonhepatic normal tissues in their low rates of lipogenesis. As observed previously (2), there was little correlation between lipogenic ability and the rate of acetate oxidation; however, comparison of the neoplastic with normal, nonhepatic tissues reveals that generally those tissues which oxidize acetate more readily also utilize more acetate carbon for lipogenesis.

In Table 2 there is demonstrated a somewhat surprising difference between the hepatoma and its normal counterpart in the tumor-bearing animal. Whereas the liver of the tumor-bearing animal displayed a very low rate of incorporation of glucose carbon into fatty acids, a property shared by liver slices of normal rats (3, 8), the tumors readily formed fatty acids from glucose carbon. That these differences represent differences in the abilities of these tissues to utilize glucose is seen from the fact that the liver also displayed a low rate of glucose oxidation as shown by the relatively low incorporation of glucose carbon in the respiratory CO_2. In contrast, the neoplastic tissue slices readily oxidized glucose; in each instance the rate of oxidation of glucose carbon was of the order of 100 times the rate of its incorporation into fatty acids.

In Table 3 several experiments are recorded in which differences were sought in the relative incorporation of acetate carbon into phospholipid and nonphospholipid fatty acids. No decisive differences were found; therefore, aside from confirming the experiments in Table 1, these experiments shed no light on the question of whether newly synthesized fatty acids appear first in phospholipids or in glycerides.

TABLE 2

INCORPORATION OF CARBON FROM UNIFORMLY LABELED GLUCOSE INTO NONPHOSPHOLIPID FATTY ACIDS OF LIVER AND HEPATOMA SLICES

Values are in conversion capacities (C.C.)

Exp. no.	Tissue	Fatty acids	Respiratory CO_2
1	Liver of hepatoma-bearing rat	0.011	6.4
	Hepatoma	0.28	34.5
2	Liver of hepatoma-bearing rat	0.02	5.5
	Hepatoma	0.46	39.3
3	Liver of hepatoma-bearing rat	0.04	8.4
	Hepatoma	0.81	55.0
4	Livers of hepatoma-bearing mice	0.01	5.6
	Hepatomas	0.71	
5	Sarcoma 37 (mouse)	0.31	37.3
6	Sarcoma 37 (mouse)	0.32	44.0

TABLE 3

INCORPORATION OF ACETATE METHYL-CARBON IN LIPIDS OF NEOPLASTIC TISSUES

Values are in conversion capacities (C.C.)

	Fatty acids			
	Phospholipid	Nonphospholipid	Nonsaponifiable lipid	Respiratory CO_2
Hepatoma, rat	0.19	0.09	0.06	4.95
Hepatoma, mouse	0.93	0.82	0.30	8.45
Rhabdomyosarcoma, mouse	0.03	0.08	0.01	1.28
Mammary tumor, mouse	0.50	0.79	0.11	8.60
Sarcoma 37, mouse	0.04	0.03	0.01	1.03

DISCUSSION

The results of this study demonstrate that tumor slices do not exhibit any extraordinary ability for lipogenesis; hence, we may conclude that the special capacity of tumor tissues for protein syn-

thesis does not extend to the fatty acid carbon chain. A rough calculation reveals, in fact, that rapidly growing tumors must obtain a large proportion of their lipids preformed from the host. For example, the rat hepatoma employed in this study reaches a diameter of approximately 1 cm. in 7–10 days. This represents about 0.5 gm. of fresh tissue, of which about 5 per cent, corresponding to 25 mg., is fatty acid. Taking the highest rate of incorporation of glucose carbon as representative, we obtain the figure, 1 micro-atom/hour/gm dry wt = 0.1 micro-atom/hour/500 mg fresh wt, or $24 \times 10 \times 0.1 = 24$ micro-atoms/10 days/500 mg fresh tissue. This figure does not represent total fatty acid synthesis but indicates only how much of the added substrate has been so utilized. In another study (2) it was estimated roughly that total fatty acid synthesis in liver slices is about 4 times the rate of acetate carbon incorporation. If we use the same factor here, we obtain a figure of about 100 micro-atoms of fatty acid carbon synthesized. This corresponds to 1,200 μg. of carbon; and, assuming a carbon content of 75 per cent, only 1,600 μg., or 1.6 mg. of fatty acid, could have been formed by the tissue. This figure is probably too high, since it is calculated from the rate of fatty acid synthesis based on the maximum weight of the tumor rather than its average weight during the growth period. Even allowing a considerable margin of error in these calculations, this value is far below the 25 mg. of fatty acid present in the tissue.

The possibility that tumors are not entirely autonomous with respect to their needs for fatty acids suggests that tumor growth may under certain circumstances constitute a drain on the lipid resources of the host. How such factors may affect the metabolism of the host and to what extent they contribute to the well known cachectic syndrome of the tumorous animal are problems which await further investigation.

SUMMARY

A study of lipogenesis in neoplastic tissues has shown that acetate and glucose carbon can be utilized for synthesis of the fatty acid chain. However, the data indicate that this process is probably too slow to supply the lipid needs of a rapidly growing tumor, and the tumor must therefore obtain its lipids preformed from the host.

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The Distribution of Stilbamidine in the Livers of Normal and Sarcoma-bearing Mice*

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In experiments preliminary to the possible use of carbon-14-labeled stilbamidine in the treatment of humans with multiple myeloma (2, 9, 26) it was found in normal mice that no abnormal concentration of stilbamidine persisted in any tissue and that over 90 per cent of the injected radioactivity was excreted within a month (20). A tracer dose of C^{14}-labeled stilbamidine was then given to a human with advanced multiple myeloma, and at autopsy 3 months later it was found that 60 per cent of the activity of the administered C^{14} was present in the liver. Because of this finding, mice bearing several types of tumors were studied; and it was found that much larger amounts of C^{14} (up to 40 per cent of the injected dose 4 days after administration) were concentrated in the liver of strain A mice bearing transplantable sarcomas and of mice with certain other tumors than in that of normal controls (27). Inasmuch as this very likely reflects important differences in the livers of normal and tumor-bearing mice, this paper describes an investigation that has been made of the site of retention of the stilbamidine in the livers of such mice. These investigations have established that the stilbamidine is bound to the mitochondria or to a particle of very similar physical properties. The binding appears to be an association or adsorption rather than chemical in nature. It does not appear to be bound mainly to the nucleic acid, as has been suggested for the stilbamidine which is deposited in the cytoplasm of myeloma cells (26).

METHODS

PREPARATION OF STILBAMIDINE-AMIDINE-C_2^{14}

4,4′-*Trans*-stilbenedicarboxamidine was prepared by a procedure modified from that reported earlier (20) and previously described methods (19) modified for tracer synthesis were employed. Potassium cyanide and cuprous cyanide were prepared as described (25).

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Cuprous cyanide-C^{14} (349 mg.; 1.94 mM; 19.6 mc.), *trans*-dibromostilbene (657 mg.; 1.94 mM) (3), and cupric sulfate (45 mg.) were sealed in a 15 × 180 mm. pyrex ignition tube together with 5 ml. of pyridine (distilled from barium hydroxide). The contents were mixed and heated with shaking for 2 hours at 250°. The bomb and contents were allowed to cool to room temperature, and then the ignition tube was cooled in liquid nitrogen before opening. Subsequently, 10–15 volumes of cold 6 M hydrochloric acid were added, and the product was removed by filtration, washed with acid, water, a small amount of cold absolute ethanol, and air-dried to yield 452 mg. (101 per cent) of 4,4′-*trans*-dicyano-C_2^{14}-stilbene.

This compound (440 mg.) was sealed with 1.5 gm. of dried ammonium thiocyanate in an ignition tube, mixed, and heated at 185° ± 5° for 14 hours. The tube was cooled in liquid nitrogen before being opened. The contents of the tube were extracted with 7–8 ml. of cold 0.5 M ammonium hydroxide and filtered. The precipitate was extracted with 10 ml. of 1 M hydrochloric acid at 80°, filtered, and extracted with 20-ml. portions of warm water, and then with acid and water again until the total volume of the extract was about 100 ml. Subsequently, the combined extracts were warmed, decolorized with 20 mg. of Norite, made 2 M in hydrochloric acid, and stored at 0° overnight. 4,4′-*Trans*-stilbene-dicarboxamidine hydrochloride was dissolved in 10 ml. of water, filtered, precipitated with ammonia, and subsequently obtained as the diisethionate. The yield was 315 mg., an over-all yield of 32 per cent from cuprous cyanide, and the specific activity was 19.5 μc/mg. From other preparations yields of 50–55 per cent have been obtained. The compound had the same infrared spectrum as an authentic sample. Paper chromatography of the product indicated that only one radioactive compound was present with the same R_F as an authentic sample (40 wt. per cent butanol-25 wt. per cent propionic acid-35 wt. per cent water was used as the solvent system).

EXPERIMENTAL METHODS

To establish the site and nature of retention of stilbamidine in the livers of the tumor-bearing mice, the following technics were used: (*a*) Fractionation with ultracentrifugation, (*b*) disintegration of mitochondria by sonic disintegration or by release of gas pressure, (*c*) enzymatic treatment of centrifuged fractions.

Liver cell fractionation procedure (*Chart 1*).—For the studies reported here, 6–8-month-old, normal or tumor-bearing (Sarcoma-1) A strain mice, maintained on a diet of Purina Laboratory Chow, were used. In each mouse of the tumor series, two sarcomas were implanted subcutaneously 7–10 days prior to

administration of the stilbamidine. At the time of sacrifice, the total tumor weight per mouse ranged from 0.8 to 6.3 gm. The mice were given intraperitoneal injections of 0.45 mg. of a freshly prepared solution of C^{14}-labeled stilbamidine diisethionate in 0.10 ml. of 0.9 per cent saline. Unless otherwise indicated, the mice were sacrificed by decapitation 96 hours after injection, and the livers were removed and fractionated by the methods described below. The mice were kept in cages designed to prevent contamination of food and water by excreta.

The livers were fractionated essentially as described by Schneider and Hogeboom (22). All operations were carried out at 0–5°, with chilled solutions of 0.25 M sucrose.

The livers were weighed, and an aliquot was removed to determine the radioactivity present by combustion and subsequent determination in the form of barium carbonate. The remainder of the liver was forced through a masher to remove connective tissue. The pulp was homogenized in approximately 10–12 volumes of 0.25 M sucrose. The homogenate (F-1) was centrifuged 10 minutes at top speed (about 2,000 r.p.m.) in an International clinical centrifuge. The sediment (F-2) of nuclei, unbroken liver cells, and red blood cells was generally rehomogenized and recentrifuged to yield the final sediment (F-2-A) and a supernatant (F-2-B), which was added to the first supernatant (F-3) and centrifuged at 9,000 r.p.m.[1] (3,440–7,350 *g.*) for 15 minutes, unless otherwise noted, to yield a precipitate (F-4) consisting mainly of mitochondria and a supernatant (F-4-S) containing microsomes and "soluble" constituents of the liver. The precipitate was rehomogenized in sucrose solution and recentrifuged at 18,000 r.p.m. (13,800–29,400 *g.*) for 15 minutes. This precipitate (F-4-P) was suspended in a suitable medium (i.e., water, 0.25 M sucrose, or phosphate buffer, pH 7.7) depending upon the nature of further experiments with this fraction. All supernatants (F-5) were combined.

DISINTEGRATION OF MITOCHONDRIA

By sonic disintegration (*11*).—The precipitate containing mitochondria (F-4-P) (Chart 1) was homogenized briefly in water, sucrose solution, or phosphate buffer (0.002 M KH_2PO_4-0.016 M K_2HPO_4, pH 7.7) and subjected to sonic disintegration for 60 minutes at 2°–4°.[2] The disintegrated mitochondria (F-D) were centrifuged at 9,000 r.p.m. for 15 minutes to yield a gray precipitate (F-D-1) and a supernatant, which was centrifuged at 38,000 r.p.m. (61,000–130,000 *g.*) for 30 minutes to yield a reddish pellet (F-D-2) and the final supernatant (F-D-3).

By release of gas pressure (*7*).—The mitochondrial precipitate (F-4-P), suspended in 0.25 M sucrose or phosphate buffer, pH 7.7, was subjected to one disintegration cycle with nitrous oxide and subsequently centrifuged to yield precipitates at 9,000 r.p.m. (F-B-1) and at 38,000 r.p.m. (F-B-2) and a supernatant (F-B-3).

Enzymatic treatment of fractions.—The mitochondrial precipitate (F-4-P) and the disintegrated mitochondrial precipitate (F-D-P) were suspended in 0.25 M sucrose and 0.02 M phosphate buffer, pH 7.3, and were subjected to the action of crystalline ribonuclease (Armour) or trypsin (Armour crystalline-50 per cent $MgSO_4$) and chymotrypsin (Armour crystalline) at 37°. Subsequently, the reaction mixture was fractionated by centrifugation.

ANALYTICAL METHODS

The measurements of radioactivity were made by direct plating of aliquots of the fractions (precipitates were resuspended by brief homogenization in water or sucrose solution, depending upon the requirements of subsequent experimental procedure), and the determinations were made with a proportional counter.[3] Self-absorption corrections were made where necessary. All determinations were made in duplicate. Checks were made by combustion and determination of the radioactivity in the form of barium carbonate by conventional technic.

Nitrogen was determined colorimetrically after Kjeldahl digestion (13). Readings were made at 440 mμ in a Beckman Model DU spectrophotometer.

Nucleic acids were precipitated in the cold by the addition of 50 per cent trichloroacetic acid (TCA) to a final concentration of 12 per cent.[4] The precipitate was washed twice with cold 6 per cent TCA, then 3 times with 95 per cent ethanol, and finally extracted with 6 per cent TCA or perchloric acid for 15 minutes at 90°. Ribonucleic acid was determined by the orcinol reaction (16, 17), and desoxyribonucleic acid by the diphenylamine reaction (5). Readings were made in a Beckman Model DU spectrophotometer at 660 mμ and 600 μ, respectively. Commercial yeast nucleic acid (Schwarz Laboratory) and sperm desoxynucleic acid (National Biochemical Corporation) were used as standards.

Succinoxidase activity was determined manometrically by the method of Schneider and Potter (24).

In all cases, results were corrected for aliquots removed for analytical purposes.

RESULTS

Site of stilbamidine-C^{14} concentration in liver cells.—The experiments indicate that a large fraction, if not all the stilbamidine[5] in the livers of sarcoma-bearing and normal A strain mice 4 days after the intraperitoneal injection of 0.45 mg. stilbamidine diisethionate is in the mitochondrial fraction.

Table 1 summarizes the results of numerous ultracentrifugal fractionations of these mouse livers. As can be seen from the table, the stilbamidine is found almost entirely in the mitochondrial fraction (F-4-P), and the distribution is the same whether the livers are from normal or sarcoma mice. About 14 per cent of the stilbamidine comes down with the slowly centrifuged nuclear fraction (F-2-A), and about the same amount remains in the final supernatant (F-5). This will be considered further.

Table 1 also shows the desoxyribonucleic acid,

[1] A Spinco centrifuge with a No. 40 rotor was used for all high-speed centrifugation (Specialized Instrument Co., Belmont, Calif.).

[2] A Raytheon 9 KC magnetostriction oscillator, type R-22-3 was used.

[3] Nucleometer, Radiation Counter Laboratories, Chicago.

[4] Unaccountably low and erratic ribonucleic acid analyses were obtained when the nucleic acid was precipitated with 12 per cent perchloric acid (23). Investigation indicated that, although essentially identical results were obtained by the use of either acid immediately after sacrifice of the mice, in the 6–8 hours required for a complete fractionation, the apparent PNA content obtained when $HClO_4$ was used as the precipitant decreased by 50 per cent or more. The desoxyribonucleic acid content obtained by either method remained essentially the same.

[5] The radioactive compound present in the liver is referred to in this paper as stilbamidine. We have only preliminary evidence that the compound present in the cell may still be stilbamidine; further work is in progress to determine definitely the chemical nature of the radioactive compound.

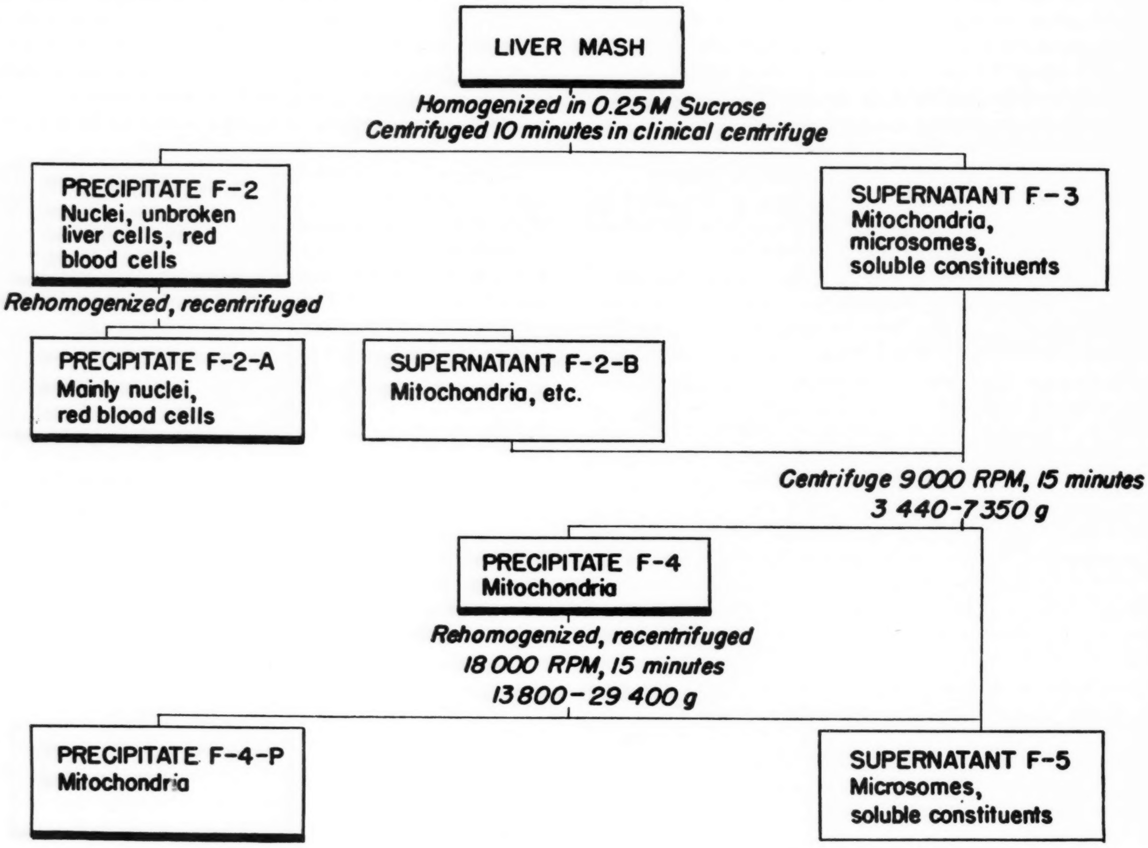

CHART 1.—Fractionation procedure for mouse liver

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN THE LIVERS OF NORMAL AND SARCOMA-BEARING A STRAIN MICE FOUR DAYS AFTER INJECTION OF STILBAMIDINE-AMIDINE-C_2^{14} DIISETHIONATE*

FRACTION	PER CENT TOTAL C^{14} IN LIVER IN FRACTION		PER CENT DNA IN FRACTION		PER CENT PNA IN FRACTION	PER CENT N IN FRACTION
	Normal mice	Sarcoma-bearing mice	Normal mice	Sarcoma-bearing mice	Sarcoma-bearing mice	Sarcoma-bearing mice
Nuclear ppt. (F-2-A)	14 (11–19)†	13 (8–20)	78 (71–83)	76 (67–83)	10 (7–15)	15 (15–18)
Mitochondrial ppt. (F-4-P)	73 (68–79)	75 (67–80)	10 (6–13)	10 (7–13)	28 (18–32)	25 (20–30)
Supernatant (F-5)	13 (7–18)	12 (8–16)	11 (10–13)	13 (10–16)	64 (52–75)	60 (54–68)
Per cent injected dose in liver	4.2 (3.0–5.5)	23 (11–31)				

* Nine normal A strain male mice, 6–8 months, average weight, 24 gm., average liver weight 1.1 gm., are represented. Twelve sarcoma-bearing A strain male mice, 6–8 months; average weight, 23 gm.; average liver weight, 1.2 gm.; and average tumor weight (two tumors), 3.7 gm. (0.8–6.3 gm.) are represented. Total N averaged 31 mg. (28–33 mg.); total DNA, 3.3 mg. (3.0–3.5) in normal mice (4) and 3.4 mg. (2.9–4.1) in sarcoma-bearing mice (6). Total PNA averaged 13.5 mg. (11.1–14. 8 mg.).

† Range.

ribonucleic acid, and nitrogen percentages in the different fractions. These values are in general agreement with those previously reported for liver fractionations (15, 21, 23, 24).

It seemed likely that the stilbamidine found in other than the mitochondrial fraction (Table 1) represented incomplete separation of mitochondria from the nuclear fraction and the final supernatant during centrifugation. In order to confirm this, a comparison of succinoxidase activity and C^{14} activity of the several fractions was made. It has been shown that succinoxidase activity resides solely in the mitochondria (12), so the finding of some succinoxidase activity in the nuclear fraction would indicate "contamination" with mitochondria. Table 2 shows that this is actually the case. The distribution of succinoxidase activity closely corresponds to the distribution of radioactivity. The fractions used for this correlation were the nuclear (F-2-A) and the two mitochondria-containing fractions (F-2-B and F-3) (Chart 1). Since succinoxidase activity resides only in the mitochondria, this indicates that the succinoxidase activity present in the nuclear fraction was due to incomplete separation of mitochondria. Furthermore, the close correlation with radioactivity percentages in these fractions is strong presumptive evidence that the radioactivity is associated with the mitochondria.

To test further the postulate that stilbamidine is associated with the mitochondria, an experiment was performed using only a non-nuclear mitochondrial fraction (F-3). Portions of this fraction were centrifuged at speeds from 1,800 to 9,000 r.p.m. The sediment and supernatant of each centrifugation were measured for succinoxidase activity and radioactivity. Table 3 shows the comparison of radioactivity and succinoxidase activity in the sediment of each centrifugation expressed as "per cent of total precipitated at 9,000 r.p.m." and indicates for each centrifuged speed the percentage of radioactivity and succinoxidase sedimented, as compared to the total precipitated at 9,000 r.p.m. It may be seen that there is a good correlation between the concentration of succinoxidase and that of stilbamidine. The largest deviation occurs in the mitochondrial sample sedimented at 3,500 r.p.m. There the sediment showed a C^{14} activity of 60 per

TABLE 2

COMPARISON OF SUCCINOXIDASE ACTIVITY AND RADIOACTIVITY IN FRACTIONS

(Results expressed as per cent of total)

	M-11*		M-13*		M-16*		M-17*		M-20*		M-65†		M-66†	
Fraction	S.O.‡	C^{14}	S.O	C^{14}	S.O.	C^{14}	S.O.	C^{14}	S.O.	C^{14}	S.O.	C^{14}	S.O.	C^{14}
Nuclear F-2-A	14	11	17	12	14	14	14	8	15	11	8	11	8	9
Mitochondrial F-2-B	13	12	18	16	17	9	9	16	15	15	5	8	10	8
Mitochondrial F-3	73	77	65	72	69	78	78	76	70	74	88	81	82	83

* A strain, sarcoma-bearing mice.
† A strain normal mice.
‡ S.O. = succinoxidase.
Total mm^3 O_2/hr consumed varied from 15–20,000 calculated for the total liver.

TABLE 3

DISTRIBUTION OF C^{14} ACTIVITY, NITROGEN, PNA, AND SUCCINOXIDASE ACTIVITY UPON CENTRIFUGATION AT SEVERAL SPEEDS

SPEED (R.P.M. FOR 15 MIN.)	RELATIVE *g*. MIN.–MAX.†	PER CENT OF TOTAL C^{14} IN PPT.	PER CENT OF TOTAL PPTED. AT 9,000 R.P.M.	NITROGEN Per cent of total ppted. at 9,000 r.p.m.	PNA Per cent of total ppted. at 9,000 r.p.m.		SUCCINOXIDASE Per cent of total ppted. at 9,000 r.p.m.	
1,800	137– 290	17	19	29				20
3,750	595–1,270	53	60	69				60
5,300	1,190–2,540	78	89	80				79
7,000	2,070–4,450	85	97	95				100
9,000	3,440–7,350	88	(100)	(100) (29 per cent of total N ppted.)				100
1,800	137– 290	15	17	11	3.2	12.7	21	25
3,500	520–1,110	53	60	65	10.2	43	68	80
4,400	820–1,750	68	76	77	12.9	49	75	87
5,200	1,150–2,440	80	90	80	16	67	79	92
9,000	3,440–7,350	89	(100)	(100)	25.8	(100)	85	(100)

* Sarcoma-bearing mice were used for the above experiments.
† Minimum and maximum refer to the *g* at the top and the bottom of the tubes.

cent versus a succinoxidase concentration of 80 per cent. Furthermore, in this experiment there is a close relationship between the total nitrogen and the radioactivity (stilbamidine) in the centrifuged sediment. Two correlations indicating association of stilbamidine with the mitochondria are thus established by this experiment: (*a*) The percentage of *succinoxidase* activity (which is found only in mitochondria) correlates very closely with the percentage of *radioactivity* (stilbamidine) in the different centrifuged fractions. (*b*) There is a close relationship between total nitrogen content of the sediments and the C^{14} activity. Since it almost certainly can be assumed that the nitrogen content of the sediment is chiefly from the mitochondria, this is good evidence that the stilbamidine is associated with the mitochondria.

The mitochondrial sediment was suspended and disintegrated. This was then centrifuged at 9,000 r.p.m. for 15 minutes producing sediment F-D-1 (Table 4). The supernatant was then centrifuged at 38,000 r.p.m. for 30 minutes producing sediment F-D-2. The supernatant of this sediment is labeled F-D-3 in Table 4.

Several interesting results were obtained in this experiment. Following sonic disintegration, as seen in Table 4, an average of 58 per cent of the C^{14} (stilbamidine) was sedimented at 38,000 r.p.m., and only 27 per cent was spun down at the slower speed of 9,000 r.p.m. About 15 per cent of the stilbamidine remained in the supernatant after the high speed centrifugation, suggesting either that this portion of the stilbamidine was detached from its binding to the mitochondria, or that this amount of stilbamidine was attached to small protein or other molecules. The fact that the great bulk of stilbamidine was sedimented indicates that there is binding with larger particulate constituents of the mitochondria. Furthermore, only 15 per cent of the ribonucleic acid content of the mitochondria remained in the final supernatant (Table 4). The inference might be, then, that the stilbamidine is combined with the ribonucleic acid in mitochondria, but this is later shown not to be the case. This also indicates that mitochondria contain particles rich in ribonucleic acid, or are surrounded by a membrane rich in ribonucleic acid, or both. About 50 per cent of the nitrogen content of the mitochondria was freed by sonic disintegration. This indicates that half of the protein of these mitochondria was released in soluble form, while the other half belonged to the ribonucleic acid-rich particles and membrane.

TABLE 4

FRACTIONATION OF MITOCHONDRIA DISINTEGRATED BY SEVERAL METHODS

Experiment	Sonic disintegration in sucrose*			Sonic disintegration in 0.02 M phosphate buffer, pH 7.7			Disintegration by gas pressure release in 0.25 M sucrose			Disintegration by gas pressure release in 0.02 M phosphate buffer, pH 7.7		
Fraction†	Per cent C^{14}	Per cent N	Per cent PNA	Per cent C^{14}	Per cent N	Per cent PNA	Per cent C^{14}	Per cent N	Per cent PNA	Per cent C^{14}	Per cent N	Per cent PNA
Sedimented at 9,000 r.p.m. F-D-1	27 (26–28)‡	11 (10–13)	9 (8–11)	24	12	8	67	31	45	66	29	23
Sedimented at 38,000 r.p.m. F-D-2	58 (52–61)	50 (40–58)	76 (73–80)	57	37	70	23	26	41	26	34	68
Supernatant F-D-3	15	39	15	17	51	20	10	42	13	8	37	9

* The figures represent the average of three experiments; sarcoma-bearing mice were used in two experiments, normal mice in the third.

† F-D-1 sedimented by centrifuging disintegrated mitochondria at 9,000 r.p.m. for 15 minutes; F-D-2 subsequently sedimented by centrifugation at 38,000 r.p.m. for 30 minutes; and F-D-3 is the remaining supernatant material.

‡ Range.

Manner in which stilbamidine is attached to mitochondria.—Preliminary experiments showed that stilbamidine is related to the liver cell mitochondria either by association or as a loose complex, because the stilbamidine can be readily extracted by dilute hydrochloric acid or ammonium hydroxide in the cold. Since stilbamidine is not soluble in cold alcohol, warm alcohol, or ether, it is not surprising that efforts to extract radioactivity with these solvents were unsuccessful. Dialysis in the cold against sucrose and against 0.9 per cent sodium chloride removed little of the radioactivity. The lability of stilbamidine to acid excluded the use of trichloroacetic acid or perchloric acid to separate the nucleic acid in order to determine whether stilbamidine is combined with the ribonucleic acid.

The problem was next attacked by breaking up the mitochondria in two ways: (*a*) sonic disintegration (11) and (*b*) disintegration by release of gas pressure (7). In both technics of disintegration, the experiments were performed on the mitochondrial sediment of differentially centrifuged liver.

Further evidence that these mitochondria consist of a membrane, particles, and an interior soluble portion rich in protein is obtained by mitochondrial disintegration by release of gas

pressure. In Table 4 it is seen that essentially the same quantity of nitrogen was released into solution as with sonic disintegration. However, it is interesting that considerably more stilbamidine and nitrogen were centrifuged down at the slow speed (9,000 r.p.m.) than following sonic disintegration. This suggests that sonic disintegration is more efficient in breaking up particles, or the mitochondrial membrane, than is release of gas pressure.

Nature of the binding of stilbamidine in mitochondria.—In an effort to determine in what manner stilbamidine is fixed to the membrane and/or particles of the mitochondria, the effect of several enzymes was studied. If stilbamidine were chemically bound to proteins, the action of proteolytic enzymes would solubilize a large part of it. Similarly, the action of ribonuclease should be effective if the stilbamidine were selectively bound to the ribonucleic acid. These studies were made on both undisintegrated mitochondria (F-4-P) and on disintegrated (sonic) mitochondria (F-D-P). The results of these studies are given in Table 5. It is seen that, for the undisintegrated mitochondria, neither ribonuclease nor trypsin and chymotrypsin significantly changed the distribution of radioactivity from that of a similarly treated sample to which no enzymes were added. The sediment of the disintegrated mitochondria treated with proteolytic enzymes contains less stilbamidine than either the disintegrated mitochondria treated with ribonuclease or the sample to which no enzyme was added. This suggests that the sonic disintegration disrupted some large aggregates containing stilbamidine to the extent that the proteolytic enzymes were able to release some stilbamidine into solution. However, it is interesting to note that even though only 15 per cent of the nitrogen was found in the sediment of the disintegrated mitochondria treated with proteolytic enzymes, 40 per cent of the *stilbamidine* was present in this sediment. *It appears unlikely, then, that there is a real chemical binding of the stilbamidine to protein.* As with the undisintegrated mitochondria, the ribonuclease had no effect on the stilbamidine content of the sediment from the disintegrated mitochondria. In all experiments there was sufficient ribonuclease action present so that, even in the undisintegrated mitochondria, only a small percentage (maximum, 22 per cent) of the ribonucleic acid remained, as determined by the conventional trichloroacetic acid method. *These results almost certainly indicate that the stilbamidine is not bound selectively to the ribonucleic acid of the mitochondria.*

Further evidence was obtained that would appear to exclude the possibility that the stilbamidine may be bound to the nucleic acid of liver. Ribonucleic acid and desoxyribonucleic acid were isolated from livers of sarcoma-bearing mice, to which stilbamidine had been previously administered, by a method in which cold saturated sodium chloride was used to extract the nucleic acid and the protein was subsequently denatured with ether (8). Following precipitation with ethanol, the nucleic acid was dialyzed and reprecipitated with ethanol. The nucleic acid isolated represented about 25 per cent of the initial ribonucleic acid present but contained less than 0.1 per cent of the initial radioactivity of the liver.

TABLE 5

EFFECT OF RIBONUCLEASE OR TRYPSIN AND CHYMOTRYPSIN ON THE DISTRIBUTION OF RADIOACTIVITY IN MITOCHONDRIAL FRACTIONS

	Undisintegrated mitochondria (F-4-P)*		Disintegrated mitochondria (F-D-P)*	
Enzyme treatment	Per cent C^{14} in ppt.	Per cent N in ppt.	Per cent C^{14} in ppt.	Per cent N in ppt.
No added enzyme	71	66	62	68
Ribonuclease (2.7 mg.)	74	62	65	56
Trypsin (2.2 mg.) and chymotrypsin (1.7 mg.)	71	41	42	15

* Six A strain sarcoma-bearing mice were used in this experiment; therefore, in each incubation the mitochondria from the liver of approximately one mouse was used. Incubations were carried out at 37° C. for 2 hours with shaking in Warburg vessels in 0.25 M sucrose and 0.01 M phosphate buffer, pH 7.3. F-D-P represents the precipitate obtained upon subjecting an aliquot of F-4-P to sonic disintegration for 1 hour at 2°–4° and subsequently centrifuging at 38,000 r.p.m. for 30 minutes. In this experiment 84 per cent of the C^{14} activity, 50 per cent of the nitrogen, and 83 per cent of the PNA were obtained in F-D-P. The percentages in the table above are those found in the precipitate upon centrifuging the incubated fractions at 38,000 r.p.m. for 30 minutes. The remainder of the activity and of the nitrogen was found in the supernatant.

Comparison of site of localization of stilbamidine with that of colloidal chromic phosphate.—It is known that colloidal chromic phosphate is quickly removed from the blood by the Von Kupfer cells of the liver (6). It seemed of interest to compare the distribution of colloidal chromic phosphate with that of stilbamidine in the centrifuged liver fractions. Five days before autopsy two sarcoma-bearing mice were given intravenous injections of colloidal chromic phosphate (400–600 μc. P^{32}).[6] The following day the usual intraperitoneal injection of stilbamidine-C^{14} was made. The results of the fractionation are given in Table 6. It may be seen that the distribution of radioactivity from the two isotopes is quite different. The stilbamidine, as noted above, is largely in the mitochondrial fraction, while the colloidal chromic phosphate is about equally divided between the nuclear fraction and the mitochondrial fraction. Intact Von Kupfer cells probably would be sedimented almost entirely in the nuclear fraction. It is likely that

[6] Dr. Ernest L. Dobson assisted with this experiment.

many of the Von Kupfer cells were broken in the macerating process. These would release their content of colloidal chromic phosphate, which it might be surmised would then be sedimented with the mitochondrial fraction. Further consideration of this is beyond the scope of this paper.

This experiment appears to indicate that stilbamidine is not handled by the liver as a colloid, although the possibility exists that the stilbamidine is collected by Von Kupfer cells and passed across the liver cell boundary into its cytoplasm and then adsorbed to the membrane of the mitochondria.

TABLE 6

FRACTIONATION OF LIVER CONTAINING STILBAMIDINE-C^{14} AND CHROMIC PHOSPHATE-P^{32}*

Fraction	C^{14} per cent of total	P^{32} per cent of total
Nuclear sediment (F-2-A)	18	43
Mitochondrial sediment (F-4-P)	71	55
Final supernatant (F-5)	12	2

* Sarcoma-bearing A strain mice were used in this experiment. They were injected 5 days before sacrifice with 400–600 μc. of $CrP^{32}O_4$, and at 4 days before sacrifice with stilbamidine-C^{14}. The P^{32} was determined by counting with a Geiger tube; C^{14} was determined after combustion to carbon dioxide.

DISCUSSION

The above experiments appear to show conclusively that stilbamidine present in the liver is nearly all localized in or on the mitochondria. This is just as true of the livers of mice with sarcomas as of normal mice. Therefore, since much larger amounts of intraperitoneally or intravenously injected stilbamidine are concentrated in the livers of sarcoma-bearing mice than in the livers of normal mice (27), it seems likely that there is a significant difference in the liver cell mitochondria when sarcoma is present elsewhere in the body.

A qualitative difference between the *soluble* proteins of the liver cell mitochondria of C3H mice and the mitochondria of Hepatoma 98/15 has been shown (10). Since the tumor used in our experiment is a sarcoma and since we have observed no metastases in sections studied, it would be interesting to determine whether the *soluble* proteins of liver mitochondria in the mice with sarcomas are also altered. Such studies are in progress. The possibility also of qualitative differences in the *non*-soluble portions of the mitochondria is suggested by our findings. Until the nature of the binding of stilbamidine is more clearly known, it cannot be decided whether the primary difference here is a physical or a chemical one.

Other evidence linking mitochondrial alterations in mice with malignancy (sarcoma and carcinoma) has recently been presented by Annau *et al.* (1). These workers have demonstrated marked histologic changes in the liver cell mitochondria of these tumor mice. These changes are characterized by a change in the size and shape of the liver cell mitochondria as compared to normal and are more marked with the more advanced tumors. It is of interest that, in our series, there is excellent correlation with tumor weight and liver concentration of stilbamidine—heavier tumors correlating with larger liver concentrations (unpublished results). We have also observed a marked increase in the concentration by the liver of stilbamidine in normal mice following heavy irradiation of the liver with colloidal chromic phosphate (P^{32}) and following liver damage with carbon tetrachloride. The stilbamidine is mostly confined to the mitochondria in the irradiated livers, as in the livers of other mice. Mice treated with carbon tetrachloride were not tested in this way. These findings suggest that some important change occurs in the function of mitochondria in response to a variety of stimuli. Whether this response in the tumor animals is produced by some toxic product of the tumor tissue or whether it is of some entirely different nature is not known.

Although others have presented evidence for a chemical binding of stilbamidine with ribonucleic acid (26), our studies indicate that, in the liver cell at least, stilbamidine almost certainly is not attached to the ribonucleic acid. The evidence strongly suggests that there is no true chemical binding of stilbamidine with any substance of the mitochondria. It seems most likely that the *stilbamidine is adsorbed to the membrane or particulate matter of mitochondria.* Evidence that mitochondria contain a membrane and inner particles has been presented by Dalton *et al.* (4). Studies with the electron microscope have also indicated a membrane and particulate bodies (28, 18). The membrane is composed of short protein fibrils and globular protein molecules, while the bodies consist of ribonuclear protein. Our studies and those of Hogeboom *et al.* (10) indicate that, in addition, the mitochondria contain a relatively large amount of protein (about 50 per cent) in a soluble form, and this is apparently not associated with the nucleic acid. The experiment described in Table 4 shows that the stilbamidine is not attached to the soluble protein, since very little was present in the supernatant which contained this protein.

In our liver fractionation experiments, the values for the distribution of nitrogen, ribonucleic acid, and desoxyribonucleic acid are in general

agreement with those reported by others (21, 23, 24); this indicates that our fractionation technic is comparable. However, our value for the percentage of ribonucleic acid in the mitochondrial fraction is somewhat higher than that reported by the above authors and may represent some contamination by the microsomal fraction. Furthermore, desoxyribonucleic acid was observed in small amounts in other fractions than the nuclear sediment. The presence of some DNA outside of the nuclear fraction is probably due to disruption of some of the nuclei. The homogenate was not ejected through a hypodermic needle, since it has been considered that this would disrupt nuclei (21).

Other experiments carried out in this laboratory have shown that a high retention of stilbamidine in liver after injection is not unique to tumor-bearing mice. Studies to be the subject of another communication show that human patients with multiple myeloma retain an unexpectedly large amount of stilbamidine in the liver and that this appears to be mostly concentrated in the mitochondria of the liver cells.

SUMMARY

The radioactivity present in the livers of normal and sarcoma-bearing mice after injection of stilbamidine-amidine-C_2^{14} diisethionate has been shown to be present primarily in the mitochondria isolated by differential centrifugation in 0.25 M or 0.88 M sucrose. Subsequent disintegration of the mitochondria by sonic disintegration, or by release of gas pressure, released a large amount of the protein and dialyzable constituents into solution but only a small amount of the radioactivity. The particulate material remaining contained most of the stilbamidine and the ribonucleic acid of the mitochondria. However, experiments with ribonuclease and with trypsin and chymotrypsin on this fraction and on undisintegrated mitochondria indicate that the radioactivity was not associated with the ribonucleic acid or with the soluble, or perhaps even with the insoluble, protein of the mitochondria. Indeed, even though no clear evidence for the exact nature of the retention of the stilbamidine has been obtained, it appears most likely to be an association, possibly adsorption, with the membrane or particulate material of the mitochondria.

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Refractoriness in the Therapy of Transplanted Mouse Leukemia*

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For specific transfer lines of mouse leukemia, survival time is increased if certain chemical agents are administered. Compounds exerting this effect are ethyl carbamate (14, 18, 20, 28), the nitrogen mustards (5), folic acid antagonists (2, 3, 4, 17, 24, 29), benzene (11, 18), potassium arsenite (11, 14, 18), competitive metabolic analogs (1, 15, 18, 19), and specific hormones (7, 37). Radiation therapy may effect similar results (10, 11, 18). The response to these agents is usually followed by a refractoriness to continued treatment with the same agent.

Resistance of specific cells to drug therapy was first observed in trypanasome infections treated with arsenicals (8). It is well known that microorganisms may become insensitive to the effects of sulfonamides and antibiotics.

Similarly, the development of resistance to the folic acid antagonist, A-methopterin, was described in AK4 mouse leukemia (6, 9), originally responsive to this agent (2). The new A-methopterin-resistant subline did not respond to other folic acid antagonists possessing a substituent 4-amino group within its molecule (8). Three sublines of lymphatic leukemia, L1210, of strain DBA, were developed (21–23). These exhibited refractoriness to A-methopterin, A-ninopterin, and A-denopterin, all closely related folic acid analogs. Resistance to one of these agents imparted resistance to the other two. The above studies suggested that resistance to treatment resided in the leukemic cell.

The problem of drug fastness may not, however, be confined to the parasitic organism or neoplastic cell. A host factor in drug resistance has been suggested (12, 13, 25).

The purpose of this investigation was to determine the probable residence of the fastness developed in the treatment of myeloid mouse leukemia with potassium arsenite and to compare biologically A-methopterin and potassium arsenite fastness. Preliminary experiments suggested that the injection of potassium arsenite into normal mice, before transplantation of line 15 myeloid leukemic cells, might inhibit the antileukemic effect of the drug. Consequently, experiments were devised to determine the contributions of the host and the leukemic cells in this particular drug resistance.

MATERIALS AND METHODS

Line 926, a transfer line of lymphatic mouse leukemia originating in the F strain, responds to therapy with the folic acid antagonist, A-methopterin (17).[1] Line 15, a myelogenous mouse leukemia, also of F strain origin, has responded to treatment with potassium arsenite (14).[2] In the latter, response was temporary in transfer generations 74–91, although, in the transfer generations up to the 32d, a majority of the treated mice did not become leukemic.

In the 68th transfer generation of line 15, an attempt was made to establish a refractory subline of cells by the following procedure. The spleen of a leukemic donor was minced and diluted with isotonic saline to contain approximately 3,000,000 cells/0.1 cc. One-tenth cc. of this suspension was injected intraperitoneally into each mouse.[3] One-half of the transplanted animals remained untreated to serve as controls. The other half were treated daily with potassium arsenite. Treatment was begun 24 hours following transplantation. When response to potassium arsenite failed to continue, as evidenced by palpable spleen and enlarged peripheral lymph nodes, the leukemic mouse was killed and used as a transplant donor.

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[1] 4-Amino-N^{10}-methyl-pteroylglutamic acid (Lederle Laboratories). Therapeutic dose was 0.05 mg. in 0.1 cc. distilled water for mice weighing approximately 20 gm.

[2] In the form of Fowler's solution, which was diluted with distilled water to contain 0.1 mg. arsenic trioxide in 0.1 cc. of this solution, was used for 20-gm. mice in all experiments, unless otherwise specified.

[3] F_1 hybrids were obtained by crossing an F or FA (derived from F) strain male with females of other strains. The F_1 generation is 100 per cent susceptible to the development of lines 15 and 926 transplanted leukemia. Although these lines arose spontaneously in the F strain, they may be grafted into the FA strain or FA F_1 hybrids.

This subline was designated line 15A. Roman numerals I, II, and so forth (Table 1) indicate the transfer generation of the subline. In this manner, line 15 leukemic cells were passed through successive generations of potassium arsenite-treated hosts. In the 101st–109th transfer generations, this procedure, designed to obtain potassium arsenite resistant cells, was repeated.

An analogous plan was followed with line 926 in which daily treatment with A-methopterin was begun 24 hours following transplantation. The development of an A-methopterin–resistant subline was instituted in the 65th transfer generation by passage through A-methopterin–treated hosts.

TABLE 1

SUBLINES 15A AND 15B OF LINE 15. THE EFFECT OF PASSAGE OF LINE 15 LEUKEMIC CELLS THROUGH SUCCESSIVE GENERATIONS OF HOSTS TREATED WITH POTASSIUM ARSENITE

(Selected transfer generations are given)

Transfer Generation	No. mice	Treatment*	Survival in day: Range	Av.	No. Negative†
			Subline 15A		
15 T 68	4	C	13–20	16.2	0
	3	P	30–105	80.0	1
15A T 69 I	2	C	17–18	17.6	0
	2	P	27–105	66.0	1
15A T 74 VI	3	C	16–22	19.6	0
	4	P	82–105	99.2	3
15A T 80 XII	4	C	36–46	39.6	0
	5	P	15–100	61.2	3
15A T 81 XIII	5	C	29–100	58.8	1
		P	32–100	68.8	2
15A T 82 XIV	4	C	36–111	56.8	0
	5	P	46–100	49.6	4
15A T 83 XV	5	C	23–100	47.8	1
	5	P	38–100	83.0	3
			Subline 15B		
15B T101 I	5	C	19–38	25.2	0
	5	P	27–47	31.8	2
15B T104 IV	5	C	18–91	41.0	1
	5	P	26–53	39.6	1
15B T107 VII	5	C	21–54	33.8	0
	5	P	37–100	67.2	2
15B T108 VIII	5	C	34–100	73.4	0
	4	P	28–49	36.6	2
15B T110 X	4	C	21–33	26.0	0
	5	P	25–48	40.0	2

* P designates animals treated with potassium arsenite following transplantation. C denotes controls which were inoculated with cells from a mouse of the previous transfer generation which had become leukemic during treatment with potassium arsenite.

† All mice surviving longer than 100 days after transplantation are listed as negative if nonleukemic at that time.

Preliminary experiments suggested that response of line 15 transplanted leukemia to arsenic therapy was altered by the injection of the drug into mice *before* the transplantation of leukemic cells. The mice seemed to develop an earlier refractoriness to treatment. Several experiments were devised to study this phenomenon.

F_1 hybrids (15–20 gm.) were given daily injections of potassium arsenite 7–67 days prior to transplantation. The mice were then transplanted with the line 15 leukemic cells. In each of the experiments all animals received 3,000,000 cells. Daily treatment with potassium arsenite was continued until death. Leukemic cells were also transplanted into a group of F_1 hybrids, half of which were carried without treatment as controls, the other half receiving potassium arsenite daily following transplantation until death.

To determine whether the fastness was relative or absolute, another group of mice received potassium arsenite for 14 days prior to transplantation, but after transplantation the dose of Fowler's solution was *increased* from 0.1 cc. to 0.15 cc. daily.

Although the trivalent potassium arsenite prolonged survival of line 15 leukemia, a gram molecular equivalent of pentavalent potassium arsen*ate* was chemotherapeutically relatively inactive. To ascertain whether the post-transplantation response to therapy was inhibited specifically by trivalent arsenic, pentavalent potassium arsen*ate* was administered for 14 days prior to transplantation. Post-transplantation treatment was with trivalent potassium arsen*ite*.

To test for a similar host resistance to A-methopterin, F_1 hybrids were injected with this drug prior to and after transplantation. Control groups were either treated with A-methopterin only after transplantation, or were untreated.

RESULTS

Potassium arsenite resistance of the host due to pretransplantation injections.—When treatment with potassium arsenite preceded transplantation of line 15 leukemic cells, the drug was ineffective in appreciably increasing survival time. Animals of this category survived only slightly longer than untreated controls (Chart 1). Only four out of 63 mice which received potassium arsenite *before* and *after* transplantation lived more than 20 days, whereas fifteen out of eighteen which received the potassium arsenite only after transplantation survived 21 days or more. The average survival of those receiving arsenite injections only following transplantation was 46 days, as compared to the control survival of 19 days. Mice treated before and after transplantation lived an average of 24 days (Table 2).

The chemotherapeutic effect was thus nullified by pretransplantation treatment with Fowler's solution. Insensitivity to therapy was manifest regardless of length of pretreatment (7–67 days), although those injected only 7 days tended to live longest.

Drug resistance was overcome when pretreated F_1 hybrids were given $1\frac{1}{2}$ times (0.15 cc.) the usual therapeutic dose of potassium arsenite following transplantation, the development of leukemia being inhibited in three of five mice inoculated with leukemic cells.

Although treatment with potassium arsenate did not increase survival time significantly (19.5 against 18.0 days in controls), pretreatment with pentavalent arsenic for 14 days prevented the response to potassium arsenite therapy. The average survival in nine pretreated mice was 22 days (range, 19–24 days), whereas seven of nine animals treated with potassium arsenite alone following transplantation outlived any of the pretreated mice, four animals never developing leukemia.

Thus, it was demonstrated that the response of

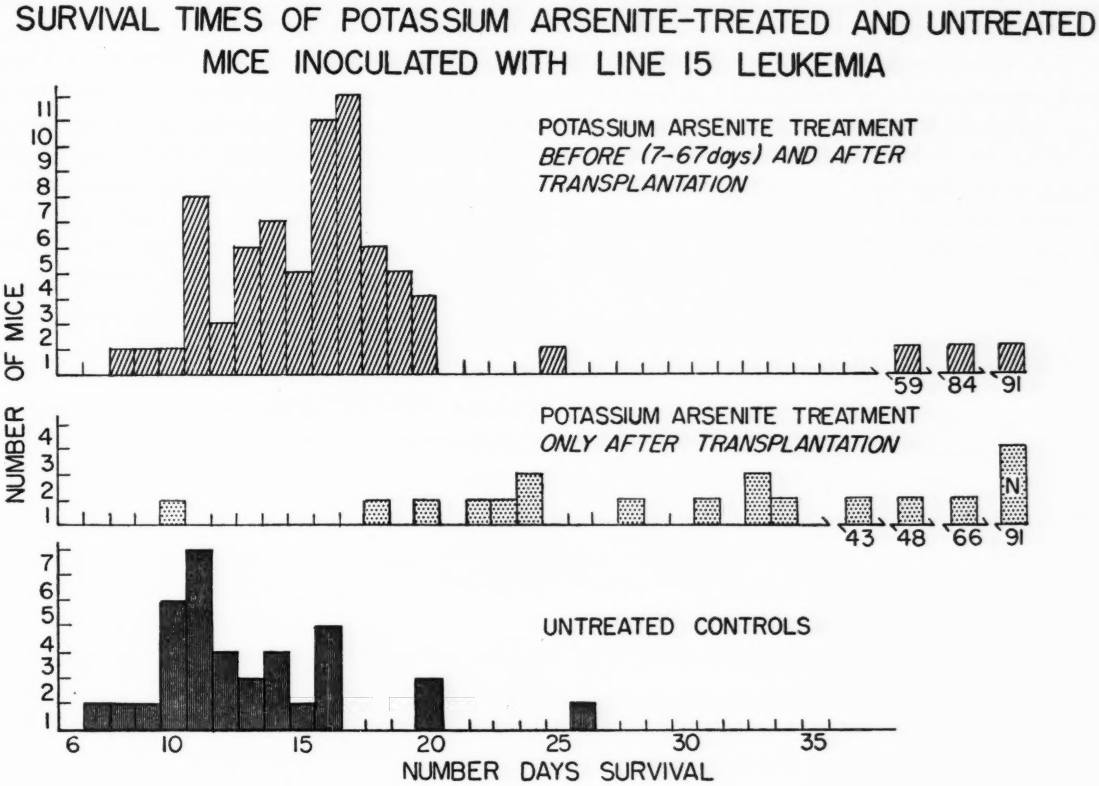

CHART 1

TABLE 2

EFFECT OF TREATMENT WITH THE DRUG BEFORE TRANSPLANTATION ON RESPONSE OF THE LINE OF LEUKEMIA TO THE SAME DRUG FOLLOWING TRANSPLANTATION

No. of mice	Survival time in days		Av. survival
	LINE 15 (POTASSIUM ARSENITE REFRACTORINESS)		
	Group I (Potassium arsenite treatment before and after transplantation)		
	20 days or less	21 days or more	In days
63	59	4	24
	Group II (Potassium arsenite only after transplantation)		
18	3	15	46
	Group III (No treatment with potassium arsenite)		
31	30	1	19
	LINE 926 (A-METHOPTERIN REFRACTORINESS)		
	Group I (A-methopterin treatment before and after transplantation)		
	21 days or less	22 days or more	In days
35	8	27	24
	Group II (A-methopterin only after transplantation)		
30	6	24	25
	Group III (No treatment with A-methopterin)		
34	34	0	11

myelogenous leukemia line 15 was nearly absent if the hosts had been injected prior to transplantation with either potassium arsen*ite* or arsen*ate*.

Lack of cellular fastness to potassium arsenite.—Attempts to produce a potassium arsenite-resistant subline of line 15 by repeated transfer of leukemic cells through treated hosts were not successful. Mice inoculated with line 15 cells passed through seven generations of arsenite-treated hosts (designated line 15A) survived longer than animals inoculated with cells that had been passed through successive generations of untreated mice (Table 1). Survival in line 15 which has been untreated ranges from 14 to 28 days, whereas in line 15A the control survival was 23 to over 100 days. However, treatment with potassium arsenite increased survival in line 15A beyond that in controls of this subline (see generations XII through XV in Table 1).

Spontaneous development of refractoriness to potassium arsenite.—Through the 35th transfer generation, treatment with potassium arsenite caused a majority of the mice to survive indefinitely without developing leukemia (14). In the 74th through the 91st transfer generations survival was con-

sistently prolonged, although the animals became leukemic. Subsequently, line 15 has been relatively refractory to the effects of potassium arsenite, but in a small percentage the development of leukemia is still completely inhibited. Line 15 retains its susceptibility to the antileukemic action of urethan to a greater degree than to arsenite, since all treated mice survived longer (25–45 days) than any of the controls (13–19 days) in the 110th transfer generation. The degree of response to

tation was 25 days. Thirty-nine controls survived an average of 11 days (Table 2).

Cellular fastness to A-methopterin.—Continued passage of line 926 leukemic cells through A-methopterin–treated hosts produced a subline which completely resisted A-methopterin therapy. The first two passages of cells responded to the agent (Table 3). In the third generation the response diminished, and, by the sixth transfer resistance to A-methopterin was complete.

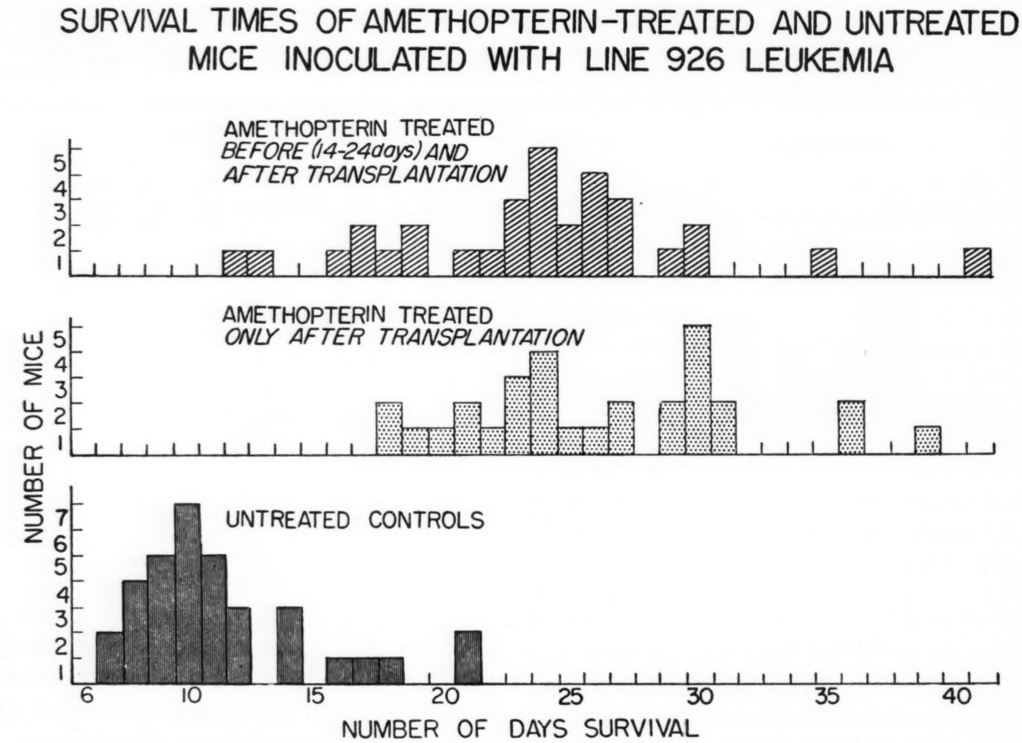

CHART 2

urethan is also diminished, however, if compared to the effect in the 10th transfer when controls survived 26 days and treated animals more than 80 days (18). Apparently, with an increase in malignancy of transfer lines during frequent transplantation, treatment highly effective in early transfers becomes less potent in prolonging life. The development of resistance to potassium arsenite during the course of transplantation has been observed for other myeloid sublines of the F strain (lines X, 686).

Lack of host resistance to A-methopterin from pretransplantation injections.—Treatment of recipients with A-methopterin prior to transplantation of line 926 leukemic cells did not alter response to the agent (Chart 2). The average survival of 32 mice treated both before and after transplantation was 24 days, while the average survival of 30 mice receiving A-methopterin only following transplan-

Mice transplanted with line 926 cells and treated with A-methopterin still respond to the drug. On the other hand, line 926 R (resistant to A-methopterin) has retained its refractoriness, even when passed through ten generations of normal, untreated hosts.

DISCUSSION

Resistance to chemotherapy of transplanted mouse leukemia may reside in either the host or the leukemic cell, depending upon the chemotherapeutic agent, the procedure used to induce refractoriness, and the line of leukemia involved. Resistance to A-methopterin was induced in the cells of line 926 R by their passage through hosts treated with the drug, whereas passage of line 15 cells through a similar number of generations of arsenite-treated hosts did not induce cellular refractoriness. Refractoriness to arsenite treatment

was induced by pretreatment of the host with the drug prior to transplantation, whereas similar host treatment with A-methopterin did not induce A-methopterin resistance of line 926 leukemia.

During the passage of line 15 through 118 generations of previously untreated hosts, a relative cellular refractoriness to the antileukemic action of potassium arsenite has appeared. As indicated above, however, the development of this resistance was not accelerated by passage of line 15 cells through arsenite-treated hosts. In the thirteenth to the 33d transfer generations, 25 of 37 treated mice survived more than 100 days after transplantation, and were considered negative for leukemia. In the 90th–95th transfers, although survival time was still increased by treatment with potassium arsenite, in only relatively few animals was the development of the disease completely inhibited. Since the type of host has been the same, the development of this refractoriness must have occurred within the cell.

The longer survival of untreated mice inoculated with leukemic cells from arsenite-treated hosts suggests a change in the cells. Survival time in line 15 (transfer generation 118) averages 18 days (range, 14–26), whereas untreated animals inoculated with cells (line 15A) from the transfers through arsenite-treated hosts survived as long as 53 days, with a few animals remaining negative. This same increase in survival time was obtained when in another experiment line 15 cells were passed through arsenite-treated hosts from the 101st to the 109th transfers (Table 1).

Host resistance to arsenite therapy of line 15 produced by pretransplantation injections might occur as a result of acquisition by the host of the ability to either detoxify or increase the rate of excretion of arsenic. Unlike the host refractoriness to arsenic demonstrated in the case of line 15 leukemia, the drug resistance exhibited by trypanasomes, plasmodia, and bacteria resides within these organisms. The trypanasomes made resistant to arsenic by continued passage through treated hosts stain differently from the arsenic-sensitive parasites (26). In the case of an antifol-resistant subline of leukemia, AK4/R, the biochemical activity in the synthesis of nucleoprotein differed from its sensitive biological homolog, AK4 (27). Up to this time, no morphologic differences between antifol-sensitive and resistant sublines have been described.

Preliminary experiments indicate that *cellular* refractoriness to x-radiation may not alone be responsible for the radioresistance of transplantable lymphosarcomas.[4] A lymphosarcoma passed through seven generations of hosts in which the tumor was locally radiated until radioresistant continued to respond to x-radiation when grafted into normal hosts. If regression was induced by systemic radiation of the host including the tumor, the results were similar.

Mice transplanted with line 15 leukemic cells and treated with pentavalent arsenic did not survive appreciably longer than untreated animals.

TABLE 3

SUBLINE 926 R OF LINE 926. THE EFFECT OF PASSAGE OF LINE 926 LEUKEMIC CELLS THROUGH SUCCESSIVE GENERATIONS OF HOSTS TREATED WITH A-METHOPTERIN

(Selected transfer generations are given. The effect on line 926 in the 156th transfer is also indicated.)

Transfer generation	No. mice	Treatment*	Survival in days Range	Av.
		926 R		
926 T 65	3	C	8–13	11.0
	3	A	19–28	23.6
T 66 I	3	C	11–14	12.6
	1	A	16	16.0
T 67 II	3	C	11–14	12.6
	3	A	17–21	19.0
T 69 IV	3	C	8–14	11.6
	2	A	12–29	20.5
T 70 V	3	C	11–17	13.3
	3	A	12–17	15.0
T 71 VI	2	C	10–13	11.5
	1	A	10	10.0
T 72 VII	3	C	10–13	11.3
	2	A	9–13	11.0
T 89 XXIV	4	A	8–13	10.7
T 91 XXVI	4	NA	8–10	9.2
T 103 XXVI	4	NA	8–15	11.0
T 104 XXVII	3	A	7–14	10.7
T 122 XLV	3	A	8–9	8.3
T 123 XLVI	3	NA	8–9	8.3
T 131 XLVI	3	NA	8.0	8.0
T 132 XLVII	3	A	7–9	7.7
T 141 LVI	3	A	7–9	8.3
		Line 926		
T 156	5	C	7–9	8.0
	5	A	13–19	16.0
T 113	5	C	9–14	11.0
	4	A	22–29	25.2

* *C* denotes controls; *A* denotes animals treated with A-methopterin following transplantation; *NA* denotes discontinuance of A-methopterin in the hosts after resistance had developed.

Pretreatment with pentavalent arsenic did, however, induce refractoriness to the antileukemic action of the trivalent potassium arsenite. It would be of interest to determine whether arsenite refractoriness is induced by the previous administration of organic arsenicals.

The development of a resistant subline of line 926 by passage through A-methopterin–treated hosts confirms the observations of others (6, 8, 9, 21, 22, 23). The resistance of the leukemic cells to A-methopterin might be explained by (*a*) mutation of leukemic cells induced by A-methopterin, (*b*) spontaneous mutation of leukemic cells coincidental with, but independent of treatment, (*c*)

[4] C. Nice and A. Kirschbaum, unpublished data.

adaptation of leukemic cells, or (*d*) selective survival of cells initially insensitive to therapy.

SUMMARY

Refractoriness of line 15 leukemia to potassium arsenite was induced by pretreating mice with the drug prior to transplantation, indicating residence of refractoriness within the host. Although line 15 cells have ultimately become relatively refractory to potassium arsenite during the course of routine passage through untreated hosts, such cellular refractoriness was not accelerated by treatment of successive generations of hosts with potassium arsenite. On the other hand, an A-methopterin–resistant subline, 926 R, was established by repeated passage of line 926 leukemic cells through A-methopterin–treated hosts. Treatment of the host with A-methopterin before transplantation did not alter the response of line 926 leukemia to that agent. These results suggest that two separate types of resistance of transplanted leukemias to drugs may be induced—one a cellular resistance residing in the leukemic cell, the other a resistance generated by the host.

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The Effect of Cortisone on the Hyperplasia Produced in Mouse Skin by Croton Oil*

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This paper describes the effect of cortisone on the hyperplasia produced in mouse skin by applications of croton oil. It has been reported that cortisone is able to inhibit mitotic activity in the epidermis of normal mouse skin but is unable to do so in epidermis rendered hyperplastic by applications of the carcinogen, 9,10-dimethyl-1,2-benzanthracene (24, 25). It thus becomes of interest to know whether this refractory state is due to some special quality of the carcinogen, or whether other agents capable of causing hyperplasia, and, in particular croton oil, would make the epidermis equally refractory. Croton oil is of special interest, for, though it is not itself a carcinogen (8, 35, 41), it is able to induce tumors in skin which has been previously treated with carcinogen, even though the carcinogen was in such small dose that, alone, it would induce only an occasional papilloma (7, 32).

Salaman and Gwynn (41) claimed that the hyperplasia produced by applying croton oil to previously untreated mouse skin differed histologically from that seen when the oil was applied to skin which had been previously treated with carcinogen. For this reason, the effect of cortisone was investigated, not only on the hyperplasia produced by applying croton oil to previously untreated skin, but also on that produced by applying it to skin previously painted with 9,10-dimethyl-1,2-benzanthracene. The cortisone was given by intraperitoneal injection, either as a single massive dose 5 hours before the mice were killed and the skin fixed or by daily injection throughout the period during which croton oil was administered.

MATERIALS AND METHODS

Adult, virgin female mice of the C strain, weighing 20–25 gm., were used. They were kept in plastic cages and fed on Rockland mouse diet, both food and water being freely available at all times.

The animals were divided into seven groups (Table 1). The carcinogen was applied to an area in the interscapular region, clipped free of hair, as a 1.5 per cent (weight/volume) of 9,10-dimethyl-1,2-benzanthracene (Eastman Kodak) in light mineral oil (Superla 34, Standard Oil of Indiana). The carcinogen was applied once only, the application being made 33 days before the first application of croton oil. The croton oil was applied to the same clipped area as a 5 per cent solution of oleum crotonis B.P. (Boots) in light mineral oil (Superla 34, Standard Oil of Indiana), applications being made twice a week until ten had been given. The single terminal dose of cortisone was of 5 mg. and was given 5 hours before the mice were killed and the skin fixed; 0.2 ml. of cortisone acetate (Merck) was injected intraperitoneally. For the daily dose,

TABLE 1

DIVISION INTO GROUPS

	TREATMENT		
GROUP	Dimethylbenzanthracene*	Croton oil†	Cortisone‡
1	None	None	None
2	None	Ten applications	None
3	None	Ten applications	5 mg. terminally
4	None	Ten applications	1 mg. daily
5	One application	Ten applications	None
6	One application	Ten applications	5 mg. terminally
7	One application	Ten applications	1 mg. daily

* 1.5 per cent 9,10-dimethyl-1,2-benzanthracene in mineral oil.
† 5 per cent croton oil in mineral oil twice weekly.
‡ By intraperitoneal injection.

1 mg. was injected intraperitoneally, cortisone acetate (Merck) being diluted with normal saline so that each ml. contained 2 mg. The dilution was made immediately before use. The first injection was given just before the first application of croton oil, and the last 5 hours before death and fixation. In order to minimize the effect of the diurnal variation in mitotic activity (11, 12, 13, 18, 22, 36), the injections were given at 9:30 A.M. and the applications of croton oil made immediately afterwards.

It should be noted that the dose of cortisone used in these experiments was large. Spain, Molomut, and Haber (44) gave mice 1 mg. twice a day for 5 days and mention no toxic effects, but Antopol (1) found that 2.5 mg. daily for 9 days caused marked toxic changes in his mice. The 1 mg. injected daily in this experiment did not seem toxic, and, in another experiment in which injections were continued for several months, did not cause loss of weight. It is, of course, a much larger dose than is used clinically, being of the order of 40 mg/kg.

The mice were killed with chloroform 5 hours after the last injection of cortisone and 29 hours after the last application of croton oil. The areas of treated skin were excised, stretched on filter paper, and fixed for 24 hours in a 10 per cent solution of formaldehyde in normal saline. They were then cut at right angles to the direction of the hair, so that three preparations

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could be made of each and blocked in paraffin. Sections cut at 6 μ were stained with hematoxylin and eosin, by the Feulgen reaction, by Van Geison's method, and in acidified 1 per cent aqueous toluidine blue; and sections cut at 10–15 μ by Verhoeff's method for elastic fibers.

RESULTS

Histological examination showed that in all the mice treated with croton oil, there was gross hyperplasia of the skin. As compared to the untreated animals, the epidermis was greatly thickened, its cells and their nuclei were enlarged and the hair follicles and sebaceous glands rendered hyperplastic. The dermis, too, was thickened, hyperemic, in places edematous, and the collagen sometimes showed the thinning described by Orr (35). The elastic tissue was scanty; the mast cells increased in number. The subdermal fat was thickened and hyperemic. There was no epilation. In none of these respects was any difference detected between the untreated mice and those injected with cortisone. These findings are in contrast to reports that, when no irritant is applied, cortisone and ACTH cause thinning of the epidermis, the dermis, and subdermal fat in the rabbit and the rat (3, 4, 6, 14), and, in the dog, rat, and man a reduction in the number of mast cells (2, 9, 14, 15).

The mitotic frequency was estimated by counting the epidermal nuclei in two of the three sections from each mouse and expressing the mitoses as a percentage of the total. The nuclei of the hair follicles and their mouths were excluded from the count. The findings are shown in Table 2. The rate in the untreated epidermis was of the order found by other investigators, who report from 0.1 to 0.3 per cent of mitoses (17, 18, 19, 23, 39). As can be seen, croton oil greatly increased the mitotic rate, the increase being as great as that reported by Cooper and Reller (19), who found 0.99 per cent of mitoses in epidermis which had been painted with 0.6 per cent 20-methylcholanthrene in benzene twice a week for 37 days, and greater than was reported by Glücksmann (23), who found only 0.36 per cent in epidermis which had been treated with 1 per cent 3,4-benzpyrene in acetone weekly for from 8 to 49 days. The increase was not so great as was found by Bullough[1] and Mottram (33), who reported that applications of 1 per cent croton oil in acetone increased the mitotic rate approximately 10 times. The data were examined statistically, analysis of variance showing that there was no significant difference between mitotic rate in the mice treated with 9,10-dimethyl-1,2-benzanthracene and those not so treated, but that the rate was higher in the mice given cortisone. The probability that the difference in rate between the animals given a single terminal dose of cortisone and those given none arose by chance was less than 1 per cent, and the probability that the difference between those given daily injections and those given none, about 2 per cent. This slight increase in mitotic rate could mean either that the injections of cortisone caused a real increase in mitotic activity or that they lengthened the mitotic cycle. In either case, the rate, as estimated by the percentage of nuclei in mitosis, would be increased.

TABLE 2

MITOTIC RATE IN EPIDERMIS

		Treatment: Ten applications of croton oil						
		No previous treatment				Previously painted once with dimethylbenzanthracene		
	No treatment	No cortisone	1 mg. cortisone daily	5 mg. cortisone terminally		No cortisone	1 mg. cortisone daily	5 mg. cortisone terminally
No. mice	6	10	9	9		5	6	4
Av.* mitotic frequency	1.3	7.3	7.9	9.5		7.5	8.3	11.1

* Number of mitoses per 1,000 epidermal cells.

It has been reported that cortisone in large doses increased the likelihood of infection in mice, rabbits, and rats (1, 16, 31, 38) and in the rabbit and the guinea pig made infection spread more widely (31, 40, 43). No such action was seen in this experiment. Intra-epithelial abscesses were seen in most of the mice painted with croton oil, but they were no more common and no more extensive in the animals given cortisone than in those not. Cortisone has also been found to reduce the inflammatory exudate in the mouse (21, 44), the rabbit (30, 31, 46), the guinea pig (34), and man (28), though perhaps not in the rat (42, 45). Many of the mice painted with croton oil showed areas in the dermis which were infiltrated with inflammatory cells, but these changes were as frequent and as marked in the mice injected with cortisone.

DISCUSSION

Parenterally administered cortisone thus failed to modify the hyperplasia produced in the skin of the mouse by applications of croton oil, except by

[1] W. S. Bullough, personal communication.

slightly increasing the mitotic rate in the epidermis. That cortisone should have little effect on epidermal hyperplasia is in accord with the findings of Nilzén (34), who found that it reduced "insignificantly or not at all" the epidermal hyperplasia induced by applying undiluted croton oil, a solution of cantharidin or a solution of colchicine to the skin of the guinea pig, and with the reports of those who studied its effect on wound healing. In the mouse (44), the rabbit (6, 10, 26, 27, 29, 37), the guinea pig (47), and man (5, 20), cortisone and ACTH have been found to have little direct effect on regenerating epidermis. Green and Savigear (25) found cortisone equally unable to reduce mitotic activity in mouse epidermis made hyperplastic by the carcinogen 9,10-dimethyl-1,2-benzanthracene, and so there is no reason to assume any difference between the reaction to cortisone of epidermis made hyperplastic by applications of carcinogen and that of epidermis made hyperplastic by croton oil, wound healing, or other means. However, in another experiment, Green and Savigear (25) found that ischemic shock decreased the mitotic frequency in the hyperplasia produced in the skin of mice by croton oil but had no effect on the hyperplasia induced by 9,10-dimethyl-1,2-benzanthracene.

As no difference was detected between the reaction to cortisone of the hyperplasia produced by applying croton oil to skin which had been previously painted with 9,10-dimethyl-1,2-benzanthracene, and that of the hyperplasia produced by applying the oil to skin not previously treated, these findings do nothing to support Salaman and Gwynn (41) in their claim that the two kinds of hyperplasia are different.

SUMMARY

1. Repeated applications of croton oil caused marked hyperplasia of the skin of the mouse.

2. Cortisone proved unable to modify this hyperplasia except by slightly increasing the mitotic rate in the epidermis.

3. The hyperplasia produced by applying croton oil to skin previously given a single application of the carcinogen 9,10-dimethyl-1,2-benzanthracene reacted in the same way to cortisone as did the hyperplasia produced by applying croton oil to skin not previously treated.

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In Vivo Inhibition of Succinoxidase Activity in Normal and Tumor Tissues by Antimycin A*

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Ackermann and Potter (1) have suggested that a preferential inhibition in cancer tissue of an enzyme common to cancer and normal tissues might be theoretically possible. This inference was drawn from *in vitro* experiments in which "irreversible" inhibitors were found to affect enzyme activity to an extent depending upon the amount of the enzyme exposed to the inhibitor. Injection of the inhibitor into the whole body would presumably inactivate most of the enzyme in a tissue low in that enzyme, while in a tissue rich in that enzyme only a small fraction would be inactivated in the absence of complicating factors such as cell permeability and variation in blood flow.

Data from earlier studies (1, 6) indicated that antimycin A might be suitable for a model study designed to test the suggestion of Ackermann and Potter, since this inhibitor was specific in action, highly effective in low concentration, and was an inhibitor of an enzymatic constituent that *in vitro* tests showed to be relatively low in tumor tissue and normal spleen, lung, and thymus, in comparison with normal heart, liver, kidney, brain, and skeletal muscle.

In the present report data are presented to show the enzyme-titrating function of antimycin A *in vivo* and *in vitro*. In addition, data are given in support of the concept that a titrating inhibitor may be *pseudo-irreversible*, as earlier defined (1), if it can be dissociated from the enzyme with the result that enzyme activity is restored. Reactivation of the enzyme has been demonstrated both *in vivo* and *in vitro* in this investigation.

EXPERIMENTAL[1]

Albino rats of specified weight and sex were obtained from the Holtzman-Rolfsmeyer Company of Madison. Tumor-bearing animals were females of this strain, which had received subcutaneous implants of tumor mince at a single site at the age of 5 weeks.

Antimycin A was administered by intraperitoneal injection of a solution in propylene glycol, unless otherwise indicated. The strength of solutions was adjusted so that between 0.15 and 0.3 ml. was injected into each rat. In control experiments, an equivalent quantity of pure solvent was administered.

The assay system for succinoxidase activity measures the rate of enzymatic oxidation of potassium succinate by tissue homogenates in a conventional Warburg apparatus at 38° C. (9). To obtain comparable rates of oxygen uptake with different tissues, 10 mg. of heart or kidney, 15 mg. of liver, 30 mg. of brain, 50 mg. of muscle, spleen, lung, or thymus, and 100 mg. of tumor tissue were assayed as water homogenates in duplicate Warburg flasks.

RESULTS

I. COMPARISON OF ENZYME-TITRATING EFFECTS *in Vivo* AND *in Vitro*

Mortality data.—Table 1 gives the mortality data obtained following the intraperitoneal injection of antimycin A into 120–150-gm. female rats. The LD_{50} value calculated from this table is 0.81 mg/kg.

Lethal doses of antimycin A usually caused death within 15–90 minutes after injection, depending on the size of the dose. Very few of the animals that survived the initial 90-minute period died later; no animals died after surviving a 24-hour period, although surviving animals were observed for 3 weeks following injection. These findings are in contrast to the results obtained by Ahmad, Schneider, and Strong by admixing antimycin A in the feed of rats (2), and indicating that a more rapid absorption of antimycin A occurs when it is administered intraperitoneally.

Injections of lethal doses of antimycin A caused air hunger that increased in severity until death.

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[1] We are indebted to Dr. F. M. Strong for generously providing antimycin A for these studies, to Dr. Asger Langlykke of E. R. Squibb & Sons for the cytochrome c, and to Dr. G. A. LePage for some of the tumor transplants.

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Some of the rats which had been injected were decapitated when at the point of death. The tissues to be tested were removed into isotonic saline, homogenized in water, and assayed for succinoxidase activity with the least possible delay—usually within ½–1 hour.

TABLE 1

MORTALITY DATA FOR THE INTRAPERITONEAL INJECTION OF ANTIMYCIN A INTO 120–150-GM. FEMALE RATS

Dose mg/kg	Deaths/no. injected
1.0	3/3
0.9	3/5
0.8	3/5
0.7	1/5
0.6	0/3
0.4	0/3

of Chart 1 was obtained by adding increasing amounts of antimycin A to a series of Warburg flasks containing a constant quantity of tissue, with the result that the succinoxidase activity of successive flasks was increasingly inhibited. These results are an extension of previously published data (6) and represent the mean of two experiments, except in the case of heart (14 experiments, mean titer 7.2, standard deviation 1.4), liver (12 experiments, mean titer 2.2, standard deviation 0.8), and Flexner-Jobling carcinoma (9 experiments, mean titer 0.48, standard deviation 0.13).

The antimycin A titer of the different tissues is considered a measure of the amount of a succinoxidase component present in the tissues. As evidence may be cited the fact that the quantity of inhibitor required to produce 50 per cent inhibition is strictly proportional to the quantity of

TABLE 2

SUCCINOXIDASE ACTIVITY OF RAT TISSUES EXCISED 1 HOUR AFTER INJECTION OF 1 MG ANTIMYCIN A/KG RAT

All values of enzyme activity are expressed as the 10/20 ratio (μl. O_2 uptake in 10 min. by 20 mg. wet tissue). Control rats were injected with an equal quantity (up to 0.2 ml.) of the solvent (propylene glycol) used for the injection of antimycin A into treated rats. Mean values are averaged from duplicate succinoxidase determinations performed with five different rats*

	Control		Treated		Statistical test of difference in means	
Tissue	Mean value	Standard deviation	Mean value	Standard deviation	Probability P	Significance
Heart	148	16	157	34	>0.6	not significant
Kidney	125	18	116	20	>0.5	"
Liver	69	8.9	28	8.6	<0.01	highly significant
Brain	32	3.5	31	2.2	>0.4	not significant
Muscle	28	4.9	26	6.2	>0.5	"
Spleen	15.0	1.2	1.6	0.4	<0.01	highly significant
Lung	9.8	1.2	2.5	1.0	<0.01	"
Thymus	10.2	2.3	8.5	1.6	>0.2	not significant
Flexner-Jobling carcinoma	8.9	1.2	8.1	0.8	>0.1	"
Walker 256 carcinosarcoma	10.4	1.3	9.6	1.6	>0.4	"
Jensen sarcoma	5.3	0.9	0.8	0.7	<0.01	highly significant

* Except control values for spleen and thymus (four rats each), treated values for lung (four rats), control and treated Flexner-Jobling carcinoma (eight rats each), and control and treated Jensen sarcoma (ten rats each). In all cases 120–150-gm. female rats were used, and results for normal tissues were obtained with nontumor-bearing normal animals.

Effect of lethal dose of 1 mg/kg.—Table 2 compares the succinoxidase activities of control tissues with those from animals injected with 1.0 mg antimycin A/kg, which has proved uniformly lethal, as shown in Table 1. The activity of each tissue was determined in duplicate, with the exception of control tissues. The significance of the results was tested by the standard *t* test (4). Succinoxidase activities of heart, kidney, brain, muscle, thymus, Flexner-Jobling carcinoma and Walker 256 carcinosarcoma were not significantly affected, but those of liver, spleen, lung and Jensen sarcoma were strongly inhibited. These results should be examined in the light of the *in vitro* evidence presented in the bottom figure of Chart 1.

The "antimycin A titer" in the bottom figure

tissue present (Chart 2) and that the antimycin A titer of different tissues runs roughly parallel with succinoxidase activity and, therefore, also with cytochrome oxidase activity and cytochrome c content (6). Hence, the bottom figure in Chart 1 illustrates the gradation in content of the affected enzyme in different tissues, as determined by *in vitro* experiments.

According to the simplest case of the *in vivo* application of the proposal by Ackermann and Potter (1), injection of the inhibitor into the whole animal should titrate the same absolute amount of enzyme in different tissues. Hence, the percentage inhibition of injected animals, as shown in the top chart, should run parallel to the *in vitro* data for enzyme content in the bottom chart, with a con-

stant amount corresponding to the enzyme titrated by the injection of antimycin A subtracted from the *in vitro* data. Such a correspondence would indicate that tissues with a relatively low enzyme content are strongly inhibited by antimycin A injected into the intact animal, while tissues with a high enzyme content are affected to a much smaller degree. The actual data show that, for the five tissues with the highest *in vitro* enzyme levels that were tested—namely, heart, kidney, liver, brain and muscle—only one, the

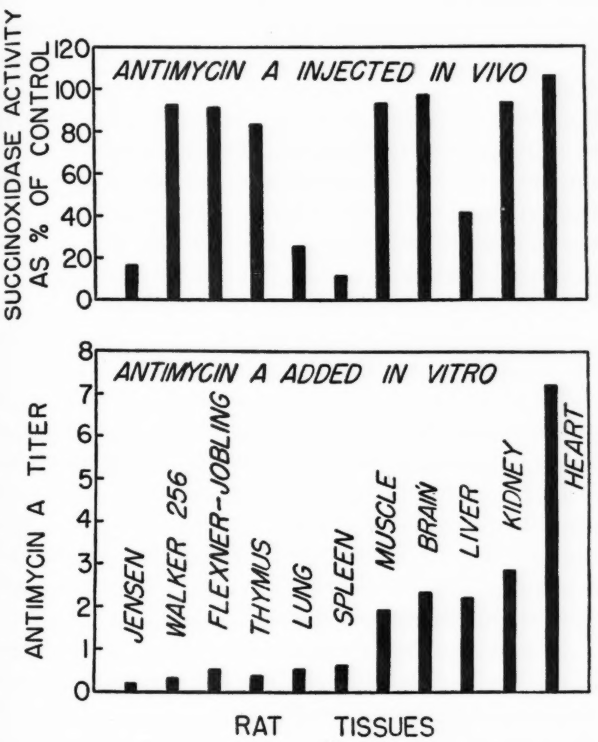

CHART 1.—A comparison of *in vitro* with *in vivo* data.

Top.—Succinoxidase activity of tissues from rats injected intraperitoneally with 1 mg antimycin A/kg rat, as per cent of the activity of tissues from control rats.

Bottom.—Antimycin A titer (μg. antimycin A required to produce 50 per cent inhibition of succinoxidase activity of 1 gm. wet weight of tissue) of various rat tissues.

liver, is inhibited *in vivo*. Of the remaining six tissues with lower *in vitro* enzyme levels, three are strongly affected *in vivo*, namely, spleen, lung, and Jensen sarcoma. Thus, liver is affected more than would be predicted, while thymus, Flexner-Jobling carcinoma, and Walker 256 carcinosarcoma are affected less than would be expected.

In contrast to the situation *in vitro*, the rate of absorption of antimycin A into individual organs *in vivo* is probably dependent on the permeability of the walls of blood capillaries to antimycin A, on the surface area of the capillaries per unit volume of tissue, and on the rate and volume of blood flow through the capillaries. However, the observed discrepancies could be explained in terms of blood supply alone, if the liver received more than a proportional share of the circulating antimycin A and if the thymus and the two tumors[2] received less.

Effects of higher doses of antimycin A.—Since the succinoxidase activity of several tissues was relatively unaffected by injections of the lethal dose of 1.0 mg., while they were known to be susceptible to the inhibitor *in vitro*, attempts were made to titrate the succinoxidase of these tissues with higher doses of antimycin. For these experiments, suspensions of antimycin A in isotonic saline were used for doses above 3.0 mg/kg. The experimental

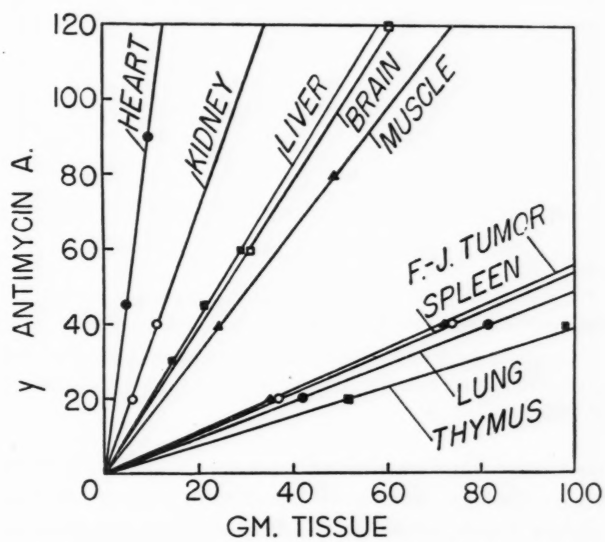

CHART 2.—Direct proportionality between the amount of antimycin A required for 50 per cent inhibition and the amount of tissue used in the test.

animals were 200–250-gm. male rats, while tumor-bearing animals were 120–150-gm. females. When the dose was increased to 3.0 mg/kg, kidney, liver, lung, spleen, and thymus were strongly in-

[2] The fact that the succinoxidase activity of Jensen sarcoma was strongly inhibited, while that of two other rat tumors was unaffected, is of considerable interest and suggested experiments to determine whether antimycin A is bound to an appreciable extent by an unaffected tumor. For this purpose, increasing amounts of antimycin A were added to flasks containing all additions for the succinoxidase system, with Flexner-Jobling tumor tissue that was excised from a rat injected with 1.0 mg/kg as source of enzyme. In three experiments, a mean antimycin A titer of 0.38 μg/gm was obtained, compared to a titer of 0.46 μg/gm for tumor tissue from control rats that had been transplanted at the same time and with the same inoculum as the injected animals. The difference in titers, 0.08 μg/gm, represents the apparent content of antimycin A in Flexner-Jobling tumor from an injected rat. This quantity of antimycin A would be insufficient to produce a significant inhibition of succinoxidase activity in this tissue.

hibited, while heart, brain, muscle, and Flexner-Jobling tumor were scarcely affected. Brain was the only tissue completely unaffected at doses up to 50 mg/kg, presumably because of the blood-brain barrier.

Intravenous injections in the tail vein at a dose of 1.0 mg/kg produced similar results to those reported above for intraperitoneal injections at 3.0 mg/kg.

Since the *in vitro* assay of tissues from injected animals could be affected adversely by the presence of antimycin in blood and extracellular fluid at the higher doses, as shown in the next section, further studies at the higher levels were not carried out. Their chief value was in showing that some tissues remained unaffected even at the higher levels, since in these cases the question of spurious inhibition could not arise.

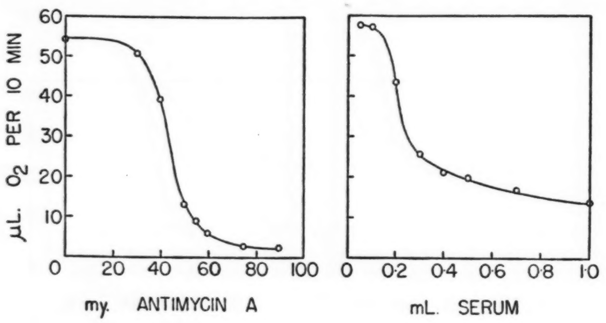

CHART 3, *a* (left).—Inhibition of succinoxidase activity of rat heart homogenate by antimycin A.

CHART 3, *b* (right).—Inhibition of succinoxidase activity of rat heart homogenate by serum from rats injected with 1 mg antimycin A/kg rat.

Antimycin A in serum from injected animals.—Succinoxidase determinations on tissues from injected animals could conceivably be affected by the antimycin A content of any blood that had been retained in excised tissues. Hence, it was necessary to measure the antimycin A content of the blood, in order to determine whether it contained sufficient antimycin A to affect the enzyme assays.

Serum was obtained from rats that were injected with 1.0 mg antimycin A/kg, and the animals were decapitated when death was imminent at approximately 1 hour after the injection. The antimycin A content of the serum was assayed by testing the inhibitory effect of various amounts, when added to a normal succinoxidase system (Chart 3, *b*). The resulting inhibition curve was compared to a standard curve (Chart 3, *a*) obtained by using known amounts of antimycin A in a test made simultaneously with the same enzyme preparation.

If the amount of antimycin A at corresponding levels of inhibition is assumed to be the same in Chart 3, *a* and 3, *b*, a series of values for the apparent antimycin A content of the serum may be obtained. When these values are plotted against the serum level to which they correspond, a smooth curve is obtained, which may be extrapolated to zero serum concentration, to give the apparent antimycin A content in the absence of serum interference with the test. A value of 0.27 μg antimycin A/ml serum was obtained by this procedure from the data in Charts 3, *a* and 3, *b*. A second experiment gave a value of 0.38 μg/ml. Three further experiments with 120–150-gm. female Flexner-Jobling tumor-bearing rats, which had been injected with 1.0 mg/kg, gave values of 0.39 μg/ml, 0.38 μg/ml, and 0.34 μg/ml. The mean value for the antimycin A content of serum from normal and tumor-bearing rats injected with the 1.0 mg/kg dose was 0.35 μg/ml. Similar experiments performed with the red blood corpuscles indicated that the bulk of the antimycin A retained by whole blood taken from injected animals was contained in the serum.

To test the validity of the described method for determining the antimycin A content of serum, a known amount of antimycin A was added to serum from normal rats to give a concentration of 0.54 μg antimycin A/ml. This serum was tested in the manner shown in Chart 3 and gave a value of 0.59 μg antimycin A/ml, which was thus within 9 per cent of the true value.

It is of interest to note the difference in shape of curves 3, *a* and 3, *b:* curve 3, *a* shows that pure antimycin A will depress enzyme activity to 5 per cent of control, while in curve 3, *b* increasing amounts of the serum from injected animals only depress the activity to 26 per cent of control. Further investigation of this difference led to the finding that antimycin A is reversibly bound by serum proteins, the main active constituent being serum albumin.[3] This binding leads to the type of curve shown in Chart 3, *b*, in which the degree of inhibition is the resultant of the reversible binding of the inhibitor by two different proteins, namely, the enzyme and the serum albumin. This effect is discussed more fully in later sections.

To estimate the effect of a content of antimycin A of 0.35 μg/ml serum on the succinoxidase activity of the tissue from an injected animal, we may examine the Jensen sarcoma, which is the tissue with the lowest antimycin A titer and therefore most likely to be affected by antimycin A content of any serum that the excised tissue has

[3] A. E. Reif and V. R. Potter, unpublished data.

retained. Assuming a serum content of 30 per cent of the wet weight of tissue (which would be unquestionably higher than is actually found) and unit density for serum, we arrive at a content of antimycin A of 0.10 μg/gm wet tissue due to serum content. Owing to the "S"-shaped form of the inhibition curve (6), 0.10 μg/gm produces less than 10 per cent inhibition of succinoxidase activity, while 0.28 μg/gm would be required to produce 85 per cent inhibition *in vitro*. It is thus apparent that the 85 per cent inhibition obtained *in vivo* is not due to the antimycin A content of serum retained in the tissue, but is probably due to antimycin A directly bound to the protein. This conclusion is likely to be even more valid for lung, spleen, and thymus, since these tissues require substantially larger quantities of antimycin A to produce 50 per cent inhibition.

II. REACTIVATION OF ENZYME *in Vivo* AND *in Vitro*

Reactivation in vivo *after sublethal doses of antimycin A*.—The previous studies with antimycin A *in vitro* (6) have shown that antimycin A behaves as a *pseudo-irreversible* inhibitor: although it titrates succinoxidase, the binding is, in fact, reversible in a system in which the inhibitor can be continually removed from the medium. The animal that survives after a sublethal dose of antimycin A is implicitly such a system, and it was of interest to determine the time course of the succinoxidase inhibition in affected organs from such animals, as well as in animals succumbing to higher doses.

Of the tissues tested at the lethal dose of 1.0 mg/kg, only liver, lung, spleen, and Jensen sarcoma had succinoxidase levels that showed a substantial reduction below control values. These tissues were accordingly tested at the sublethal level of 0.6 mg/kg, to determine whether these tissues were still affected at this dose. Chart 4 summarizes the results obtained when rats were sacrificed at stated time intervals after injection with antimycin A, and the succinoxidase values of liver, lung, and spleen were assayed immediately following sacrifice. Values obtained from injected animals are compared with control values, to which an activity of 100 per cent is assigned.

In the top and middle charts, all three tissues that were assayed were excised from the same animals, and corresponding tissues from two animals were pooled for assay. Each point in the top chart represents the mean value of assays on six rats, while in the middle chart each point represents the mean value from four rats. In the bottom chart each point represents separate assays on a minimum of four rats, and 112 rats were used in all for its construction. The degree of variability of control values is exemplified by the data for spleen of the bottom chart, where the results of fourteen separate assays gave a mean Q_{O_2} value of 18.5 μl O_2/10 min/20 mg dry tissue, with a standard deviation of 1.4, or 8 per cent. The highest deviation of results with tissues from injected animals was obtained at that point of the curves where there was a rapid change in activity with time, such as at the 1-hour point for the 0.6 mg/kg dose in the bottom chart, where results with seven rats showed a standard deviation of 18 per cent.

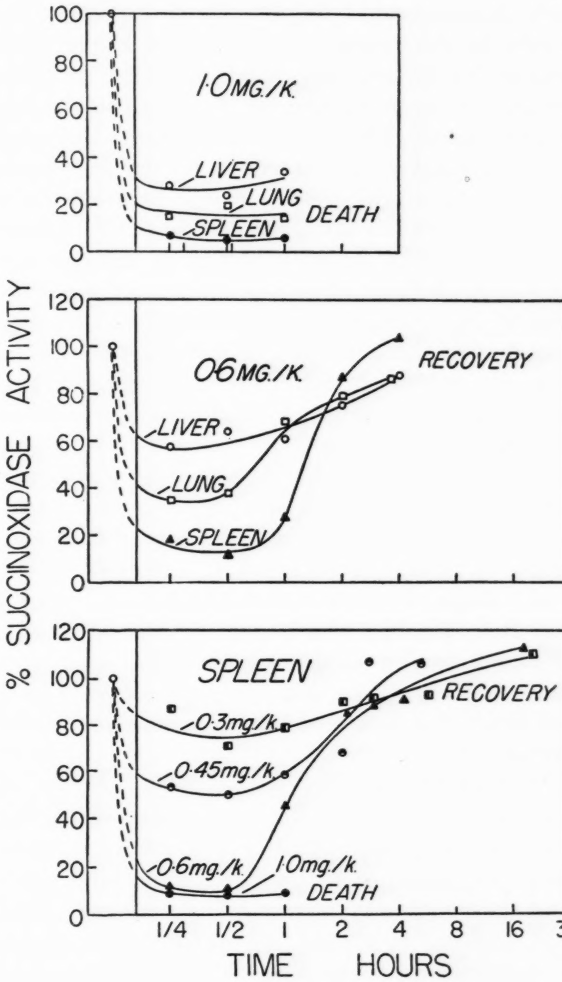

CHART 4.—Effect of the intraperitoneal injection of antimycin A on the succinoxidase activity of spleen, lung, and liver of the rat, as compared to control values.

Top chart.—Effect of the minimum lethal dose of 1.0 mg/kg.

Middle chart.—Effect of the maximum tolerated dose of 0.6 mg/kg.

Bottom chart.—Effect of different doses on the succinoxidase activity of spleen.

There is no repetition of data in any graphs: the curves for spleen at 1.0 mg/kg that are shown both in the top and middle charts were obtained

separately with different animals; this also applies to the curve at 0.6 mg/kg, given in both middle and bottom charts. The degree to which these two sets of curves agree is a measure of the reproducibility of the results.[4]

The top chart shows that *lethal* doses of antimycin A cause rapid depression of succinoxidase activity in the tissues studied. The activity remains at the initially low levels until the rat dies, normally between 60 and 90 minutes after injection of this dose. The middle chart shows that at a high *sublethal* dose of antimycin A, the succinoxidase activity of the tissues drops to levels only slightly higher than those reached at a lethal dose. However, enzyme activity begins to recover approximately ½ hour after injection, and recovery is practically complete after 3 hours. This recovery is not due to the synthesis of new enzyme but is due to the release of inhibitor from the enzyme, since the release can be accomplished *in vitro*, as shown in a later section.

The bottom chart shows the effect of varying the doses of antimycin A on the succinoxidase activity of a single tissue, spleen, that was chosen because it is the most strongly affected of the tested tissues. It is seen that slightly lower sublethal doses produce much smaller effects; thus, a dose of 0.3 mg/kg caused an inhibition of succinoxidase activity that was only just significant, while twice this dose, 0.6 mg/kg, was already the maximum tolerated dose.

The recovery of enzyme activity shown in these charts paralleled the abatement of visible physiological symptoms after the administration of sublethal doses of antimycin A. Similarly, continued depression of enzyme activity corresponded to the increase of visible physiological symptoms and resulted in death. The data, therefore, suggest that continued function of oxidative enzymes in vital tissues other than heart and brain is essential for the survival of the animal.

Effect of sublethal doses on Jensen sarcoma.—Of the three tumor tissues tested at a level of 1.0 mg/kg, only Jensen sarcoma had succinoxidase values that were strongly depressed. In this tissue the reduction amounted to 85 per cent (Table 2). Since any therapeutic trials would have to be made with sublethal doses of antimycin, it was essential to measure the succinoxidase levels in surviving animals. The results obtained at a sublethal dose of 0.6 mg/kg are recorded in Table 3.

TABLE 3

SUCCINOXIDASE VALUES OF JENSEN SARCOMA EXCISED AT VARYING TIMES AFTER INJECTION OF 0.6 MG ANTIMYCIN A/KG RAT*

	Control	Injected		
Time after injection (min.)	—	20	60	150
No. of rats	8	6	6	6
Mean 10/20 ratio	5.4	4.2	3.1	3.8
Standard deviation	1.9	2.1	1.8	1.8
Probability P	—	>0.2	<0.05	>0.1
Significance	—	Not significant	Significant	Not significant

* All values of enzyme activity are expressed as the 10/20 ratio (μl. O_2 uptake in 10 min. by 20 mg. wet tissue). Control rats were injected with an equal quantity (up to 0.2 ml.) of the solvent (propylene glycol) used for the injection of antimycin A into treated rats. Mean values are averaged from duplicate succinoxidase determinations performed on each tissue.

The results show a high degree of variability.[5] The succinoxidase values in control and injected animals had standard deviations of 28 and 52 per cent, respectively. These high deviations made it essential to use statistical means to test the significance of the results. When the standard *t* test (4) was used for this purpose, it was found that the results were not significant for two of the time periods studied, 20 and 150 minutes; but for the 60 minute period the results were significant. However, if these data are averaged for all three time periods and compared to the control, they become highly significant. These data establish that a single sublethal dose of antimycin A inhibits the succinoxidase activity of Jensen sarcoma to some degree. Repeated sublethal doses would, therefore, probably have a more pronounced effect.[6]

[4] Experiments were done to investigate whether the time interval between excision and assay had any effect on the enzyme activity of different tissues at the critical points of Chart 4 where enzyme activity was changing rapidly with time. For this purpose, a rat was injected with a dose of 0.6 mg/kg, and sacrificed at 1 hour, when the liver, spleen, and lung were excised into isotonic saline at 0° C. and a portion of each tissue homogenized and assayed for succinoxidase activity. The remaining portions of the tissues, as well as the corresponding homogenates, were permitted to stand at room temperature for 2 hours before being similarly assayed. Some reactivation of enzyme activity in whole liver was observed, while liver homogenates precisely maintained their enzyme activity over the storage period. With spleen and lung, a depression of enzyme activity occurred both in the intact and in the homogenized tissue. Further study showed that under these conditions one-half the reactivation observed with whole liver occurred within the first ½ hour. In the course of actual experiments, these effects were held to a minimum by homogenizing and assaying tissues as soon as possble after excision.

[5] The Jensen sarcoma was used as subcutaneous implants that were taken 6 days after implantation when used for enzyme studies. Even at this early stage, the tumors were usually either necrotic or hemorrhagic, and it was sometimes difficult to obtain sufficient healthy tissue. These factors may have contributed to the high variability of the results.

[6] In particular, it was of interest to investigate whether repeated doses would affect the growth of Jensen sarcoma, in order to establish whether there was any correlation between partial inhibition of succinoxidase activity and survival of the tumor. To this end, antimycin A was injected at the dose of 0.3 mg/kg twice a day for a total of seven injections, without obtaining a significant difference between con-

Reactivation in vitro *by normal rat serum.*—As the data in Chart 3, *b* suggest and further experiments have proved,[3] normal serum is capable of reversing the inhibition of succinoxidase after the addition of antimycin A *in vitro*. This fact suggested a means for determining whether the restoration of enzyme activity *in vivo* (Chart 4) in surviving rats was due to a removal of the inhibitor from a reversibly titrated enzyme or to the regeneration of new enzyme following destruction of the enzyme originally present.

Chart 5 presents data from an experiment in which tissues were removed from two rats 1 hour after the injection of 1 mg antimycin A/kg. The succinoxidase assay was carried out with 0.3 ml. of normal rat serum in the side-arms of the Warburg flasks. After a preliminary measurement of the enzyme activity in the absence of serum (which confirmed the data in Chart 4), the serum was added from the side-arm, and the rate and extent of restoration of enzyme activity were noted, as shown in Chart 5. The control values were obtained from the tissues of a normal rat tested simultaneously to show the approximate level of activity that the treated tissues might be expected to attain.

These data show the feasibility of reactivating tissues from injected animals for use as their own controls in experiments of the type reported in Chart 4. In addition, these results indicate that the *in vivo* inhibition of enzyme activity by antimycin A, shown in Chart 4, is reversible *in vitro* and presumably also *in vivo* by a mechanism involving the binding of antimycin A by the serum proteins. Bound antimycin A may be carried to the liver and there inactivated, since *in vitro* experiments have shown that liver was the only one of nine tissues tested that was capable of inactivating antimycin A (6).

Reactivation in vitro *by normal cell fractions.*—Since it was clearly established that homogenates from animals injected with antimycin A contained succinoxidase combined with antimycin A in a form that could be removed by adding another protein with a high affinity for the inhibitor, it was of interest to test whether the succinoxidase could be dissociated from the inhibitor simply by centrifuging the particulate enzyme and resuspending it several times. Homogenates in isotonic KCl were centrifuged at 5,000 *g* for 10 minutes. The residue was resuspended in KCl and the procedure repeated. The final "washed residue" containing nuclei, mitochondria, and some microsomes was then tested for succinoxidase activity.

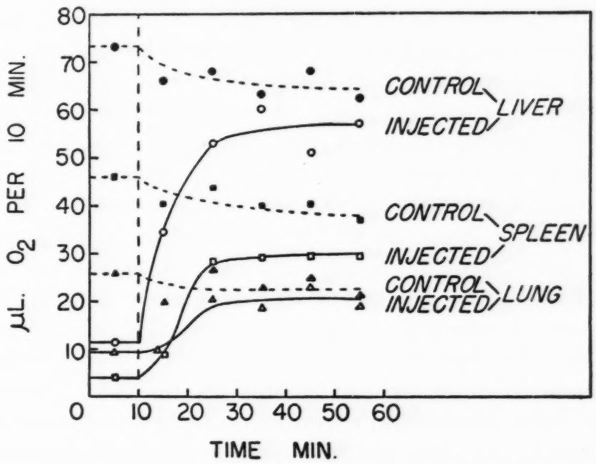

CHART 5.—The reactivation of succinoxidase activity of affected tissues from two rats 1 hour after injection of 1.0 mg antimycin A/kg, obtained by tipping 0.3 ml. normal rat serum into the assay flasks 10 minutes after the start of oxygen uptake readings. Data obtained with tissues from a normal rat are included for comparison.

In two experiments, the final "washed residue" had succinoxidase activities of 18 and 31 per cent, respectively, of the original activity in the liver homogenate from the injected animals. When this procedure was repeated with liver from a normal animal, the "washed residue" had 85 per cent of the activity of the whole homogenate. Thus, washing liver particulates from injected animals not only failed to remove the inhibitor, but the degree of inhibition was actually *increased.* This indicated that the portion of the homogenate removed during this procedure, namely, the supernatant from the mitochondria, tended to counteract or bind antimycin A. Similar results were obtained with homogenates of spleen from injected animals.

To test this idea, rat liver from normal and injected rats was fractionated in 0.25 M sucrose by a procedure based on the method of Schneider and Hogeboom (8). It was found that mitochondria from injected animals had 16 per cent of the succinoxidase activity of normal rat mitochondria. However, this activity could be raised to 62 per

trol and injected rats (six animals). At a dose of 0.4 mg/kg, repeated doses were found lethal to a percentage of the animals unless restricted to a single dose administered on no more than 5 successive days. At this dosage, an apparent decrease in tumor size of treated animals was obtained. In three experiments with a total of 23 animals, the mean tumor weights of treated rats were 31 per cent, 40 per cent, and 91 per cent of that of the controls. However, when these results were tested by the *t* test (4), all three results were found to be statistically insignificant. In two experiments (ten animals) at a level of 0.5 mg/kg, five doses over a period of 3 days resulted in death of treated animals; however, the tumor weights in treated animals, taken within 14 hours of death, were 53 per cent and 19 per cent of that of controls. It is concluded that the data presented fail to show a statistically significant effect of antimycin A on growth of Jensen sarcoma.

cent on addition of a small quantity of normal microsomes, that alone had negligible succinoxidase activity and that raised the succinoxidase activity of normal mitochondria by only 9 per cent. The addition of the same quantity of microsomes from an injected animal raised the activity to only 25 per cent of the activity of normal mitochondria. These data show that the succinoxidase activity of mitochondria from the liver of an injected animal can be reactivated *in vitro* by constituents of normal liver.

DISCUSSION

The term *pseudo-irreversible* was introduced by Ackermann and Potter (1) to describe the action of enzyme inhibitors with two characteristic features. In the first place, they must combine with the enzyme inhibited with an affinity that is so great as to give the appearance or practical effect of irreversibility; that is, there must be a titrating effect in which the amount of enzyme inactivated will depend not only upon the amount of inhibitor but upon the amount of enzyme present. Secondly, a pseudo-irreversible inhibitor must in fact *not* combine irreversibly with the enzyme or otherwise destroy it, but must have a true dissociation constant (albeit extremely small), so that the dissociation of the enzyme-inhibitor complex results in an enzyme with unimpaired activity. The demonstration of the latter condition has been realized experimentally by introducing to a pseudo-irreversibly inhibited enzyme system an *additional component* that has a high affinity for the inhibitor. As the added component associates with the traces of free inhibitor in the medium, the enzyme-inhibitor complex dissociates, and the activity of the enzyme is restored.

The action of irreversible or pseudo-irreversible enzyme inhibitors is of particular interest in connection with problems encountered in cancer chemotherapy. The present report has dealt with observations on antimycin A administered *in vivo*. While this substance may have no practical possibilities as a chemotherapeutic agent against cancer, the experiments serve to illustrate principles of fundamental significance.

Antimycin A behaves as a pseudo-irreversible inhibitor with a high degree of specificity. The amount required to produce 50 per cent inhibition is directly proportional to the amount of tissue in the system (Chart 2), while it evidently combines with relatively few components in the tissues, since the effective amount is not only very small but varies widely from one type of tissue to another (6) and is closely related to the amount of the cytochrome system in the tissue (6). The second requirement for demonstrating pseudo-irreversibility is also met, since, by the simple addition of a few tenths of a ml. of rat serum (Chart 5) or a few milligrams of crystalline serum albumin,[3] the activity of a completely inactive enzyme system can be restored. Similar restoration of activity has been observed in liver homogenates without addition of extra components that bind the inhibitor, presumably owing to the metabolism of the inhibitor (6).

The *in vitro* data are of considerable help in explaining the *in vivo* experiments. In the first place, the fact that the inhibitor is pseudo-irreversible in action makes it possible to inject the inhibitor into the whole animal and to determine the effective amount of enzyme remaining in various tissues after they have been excised and transferred to *in vitro* test systems at high dilution. The fact that the enzyme is inhibited in some tissues and not in others is largely a matter of the relative amounts of enzyme in the tissues (Chart 1), but the variations in blood flow per unit of tissue, and the permeability barriers modify the result. The fact that the enzyme activity is low in the affected tissues and returns with time in the animals that survive (Chart 4) might have been explained as destruction and resynthesis of the enzyme; but the reversal with serum *in vitro* (Chart 5) and the other available information (6)[3] show that the enzyme is not destroyed but is in tight combination with the inhibitor. The reversal in the surviving animals is explained in terms of the metabolism of the inhibitor, probably in the liver (6), with a continuous transfer of the inhibitor from the tissues to the liver via the serum albumin[3] until the tissues have been released from the inhibition.

The correlation between survival and release from inhibition, and the high degree of inhibition of the succinoxidase system in spleen and lung in nonsurviving animals in which the enzyme activity in heart and brain is scarcely affected, suggest that these animals do not die because of primary damage in heart or brain, although damage to unknown enzymes is not precluded.

The present data have demonstrated that the inactivation of an enzyme in several organs without the inactivation of the same enzyme in other organs is a practical possibility. In the chemotherapy of cancer, it would be necessary to use an inhibitor that at sublethal doses would strongly depress the activity of a vital enzyme in the tumor without irreparable damage to normal tissue. If this enzyme is unique to tumor tissue, the problem would be relatively simple; if the enzyme is common to both normal and tumor tissues, the present

data show that the enzyme would have to be present in lower amounts in the tumor tissue, or, alternatively, other factors would have to provide a more effective delivery to the enzyme in the tumor. Intrinsic factors, such as blood supply of the tumor, permeability to the inhibitor, and binding of the inhibitor by the tumor, would greatly influence the results. Extrinsic factors, such as attempts to slow down the removal of inhibitor from the tumor once it has been delivered (e.g., by lowering blood pressure [3]) or to carry out a sequential block (5) in a system in which the tumor lacks alternative pathways, might be used to intensify the effect on the tumor. The rational application of these principles may require a more extensive knowledge of the intermediary metabolism of tumors than is available at present.

SUMMARY

1. Experiments were done to test the theory that the injection of a titrating inhibitor into the intact animal should strongly affect enzyme activity in tissues low in the enzyme, while tissues high in enzyme content should be relatively unaffected. The results obtained show a tendency in accord with this theory. The large discrepancies observed with several tissues are possibly related to differences in blood supply and differences in permeability to the inhibitor.

2. Reactivation of succinoxidase activity in liver, spleen, and lung was observed *in vivo* following injection of sublethal doses of antimycin A. The enzyme activity of these tissues was strongly depressed for the first 30 minutes after injection, but the enzyme was fully reactivated within 3 hours in surviving animals.

3. Reactivation of succinoxidase activity in tissues from injected animals was obtained *in vitro* by adding normal rat serum or normal cell fractions, indicating that reactivation *in vivo* was accomplished by transfer of the inhibitor from the enzyme-inhibitor complex to serum albumin, followed by dissociation from serum albumin concomitantly with inactivation or excretion of the inhibitor.

4. The results were discussed in terms of the concept of *pseudo-irreversible* enzyme inhibitors, which are defined as inhibitors that have the effect of titrating the enzyme with which they react and that nevertheless form a dissociable complex with the enzyme.

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The Induction of the Shope Papilloma in Transplants of Embryonic Rabbit Skin*

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One of the problems incident to the successful transplantation of embryonic tissue concerns the status of the transplant in relation to the constitution of the host. Variations in susceptibility to infectious agents offer a convenient measure of constitutional difference and have been employed for this purpose in the study of homologous transplants in immunologically altered hosts as well as of heterologous transplants between susceptible and resistant species. A variety of agents of both viral and bacterial nature has been used, and the results to be reported are representative of the wide, general susceptibility found to characterize embryonic transplants.

The present paper is concerned primarily with the susceptibility of embryonic rabbit skin to the Shope papilloma virus in adult animals of homologous and heterologous species, but in as much as the results of control experiments involving the transplantation of domestic papilloma tissue in such hosts are at variance with the findings of other laboratories, as well as with comparable studies of other benign tumors, this phase of the investigation will be given some emphasis.

MATERIALS AND METHODS

The stock material used for inoculation consisted of glycerinated papillomatous tissue derived from cottontail rabbits. Fragments of this tissue were washed in saline, ground in a homogenizer, and diluted with saline to a 5–10 per cent cellular suspension. The suspension was allowed to stand until the larger fragments had settled and the supernatant fluid appeared clear and colorless. In some experiments the supernatant fluid was used directly, while in others a cell-free filtrate was obtained by passage through a Type N Berkfeld filter.

The rabbits were of Dutch, Himalayan, or New Zealand breed, the mice of the DBA strain, and the guinea pigs of mongrel stock obtained from commercial breeders.

Rabbit embryos were obtained from pregnant does killed between the seventeenth and 21st days of gestation. In some cases the skin was removed in fragments measuring approximately 1 mm. in diameter and placed in the virus solution. In others, an area of skin *in situ* was scarified with a sharp knife and dissected in block. The skin was laid flat in a petri dish with its epithelial surface uppermost and covered with the virus fluid. After soaking in the fluid for from 30 minutes to an hour, small fragments were cut for transfer. The fragments were then transplanted to the brain or anterior chamber of the eye of adult animals using technics described in previous reports (4, 5).

RESULTS

Induction of papillomas in embryonic rabbit skin with cellular suspensions of cottontail papillomas.—Six different groups of rabbit embryos ranging from 17 to 21 days of intra-uterine age were used in these experiments. The skin was dissected, and, after immersion in the papilloma preparation, fragments were transferred to 39 adult rabbits. The brain was used as a transplantation site in 33 cases and the anterior chamber of the eye in six.

The general behavior and growth rate of the anterior chamber transplants did not distinguish them from normal embryonic skin throughout the first 2 weeks of their course. At that time, however, white opaque areas appeared and rapidly increased in size to occupy the greater part of the transplant by the 20th day. Histological examination in all cases disclosed a cystic mass lined by hyperkeratotic, papillary epithelium and filled with keratin (Fig. 4).

The majority of rabbits bearing brain transplants showed no neurological signs, but occasion-

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ally animals were found dead or comatose by the fifteenth day. One rabbit was held under observation for a period of 3 months, but all others were killed between the seventeenth and 40th days.

In all instances, the brains contained rounded masses of tissue measuring from 0.5 to 2 cm. in diameter and sometimes occupying the greater part of a cerebral hemisphere (Figs. 1, 2A, and 2B). Occasionally, long tufts of hair extended from the transplants through the ventricle to appear on the external surface, but as a rule such transplants showed only isolated areas of papillomatous transformation. The usual tumor mass consisted of a central core of soft, flaky keratin surrounded by firm friable tissue and bore no resemblance to normal skin.

Histologically, all the transplants showed complete or partial transformation into structures morphologically identical with the papillomas found in adult animals. In the majority of cases the entire transplant was involved, and normal epidermis was not found (Fig. 7). The amount of keratinization varied with the age of the transplant—early transplants consisting almost wholly of papillomatous masses of squamous epithelium, while old transplants were generally cystic with a large core of keratin and a lining of hyperkeratotic papillary epithelium. As noted above, occasional transplants contained normal epidermis and hair follicles interspersed with areas of papilloma formation (Fig. 6).

Induction of papillomas in embryonic rabbit skin with cellular suspensions of domestic rabbit papillomas.—The experiments described in the previous section were duplicated utilizing suspensions of adult domestic papillomas as the infectious material. The results were less consistent than those obtained with the cottontail papilloma, but sufficient growths were elicited in transplants to demonstrate that the adult material used was infectious for embryonic skin. In contrast, the application of the same suspensions to the scarified skin of adult domestic rabbits failed to induce any changes.

The findings were inconstant in relation to both occurrence and stage of development. As a rule, no more than a half of the group of rabbits used in an experiment bore transplants containing papillomas. Furthermore, advanced papillary transformation was sometimes present as early as the fourteenth day (Fig. 16), whereas other transplants, subjected to treatment with the same emulsion, showed only early proliferative changes on the 35th day. The domestic papillomas utilized for infectious material varied from 20 days to $2\frac{1}{2}$ months in age, but there was no indication that age was concerned in the variation. The storage of papillomatous tissue in glycerine did not appear to enhance its infectivity and in several experiments gave more variable results than when used in a fresh state.

It seems probable that a uniform infection of embryonic skin fragments was not obtained and that, in the negative case, the technic utilized was not adequate to insure contact between the epithelial cell and the infectious agent. Present experiments are concerned with variations in the methods employed with the hope that some modification may lead to a greater constancy of results. It should be emphasized, however, that despite the variability encountered the experiments demonstrate that the infectiousness of the Shope papilloma virus for embryonic skin is not lost after residence in an adult domestic rabbit.

Serial induction of papillomas in embryonic rabbit skin utilizing cellular suspensions.—In this series of experiments normal embryonic skin was bathed in cellular suspensions of papillomatous tissue induced in brain transplants of embryonic skin and then transplanted to a second generation of rabbits. This series involved six papillomas induced in embryonic brain transplants, and the fresh embryonic skin treated with suspensions of the papillomatous tissue was transferred to the brains of eighteen rabbits.

The eighteen rabbits were killed for histological examination of the transplants between the seventeenth and 35th days after transfer. All the transplants obtained after the 31st day showed typical papillomatous transformation (Fig. 8). In some instances characteristic proliferation was found as early as the seventeenth day, but the majority of young transplants showed only suggestive changes or no alteration from normal. It was apparent that the period of time required for papilloma development was longer than needed when cottontail material was used as the infectious material.

A third serial transfer, utilizing fresh embryonic skin and a suspension of the second generation of induced papillomas gave rise to papillomas after a comparable period of time.

Serial transplantation of tissue from papillomas induced in embryonic rabbit skin.—The successful serial induction of papillomas in the embryonic skin of domestic strains of rabbits contrasts sharply with the results obtained in the intact skin of adult domestic rabbits. Papillomas are readily induced in such animals with material obtained from cottontail rabbits, but attempts to induce papillomas in a second generation of domestic rabbits generally fail. Control experiments were undertaken, therefore, in an attempt to interpret the

significance of this variation. Despite the reported failure of attempts to transfer papillomatous tissue from one domestic rabbit to another by direct grafting technics, the possibility remained that living cells in the emulsion might adhere to the embryonic skin used for transfer and, either because of differences in biological activity or because of a more favorable transplantation site, might grow and thus simulate the induction of a new papilloma. Accordingly, experiments were carried out to determine the transplantability of papillomas induced in embryonic skin. Other control experiments, including the use of filtrates rather than cellular suspensions, will be described in a later section.

Papillomatous tissue was removed from transplants in the brain under sterile conditions, and fragments were transferred to the brains of second generation domestic rabbits. Five different papillomas were tested in this manner, and twenty adult rabbits were used as hosts. These animals were killed at intervals between the seventeenth and 35th days, and all but five bore growths. The transplants had increased from fragments 1 mm. in diameter to masses measuring from 1 to 2 cm., and on microscopic section they were found to be made up entirely of papillomatous epithelium and keratin (Fig. 9).

Third generation transfers were carried out in several cases, and, although the percentage of takes decreased and the resulting growths were considerably smaller, microscopic examination showed typical papillomatous tissue.

Serial transplantation of tissue from papillomas induced in adult rabbit skin.—In an attempt to determine whether the transplantability of papillomas induced in embryonic skin represented a unique property differentiating them from growths induced in adult skin, or whether the reported failure to transplant adult papillomas related to an unfavorable transplantation site, a series of experiments was instituted with duplication of the conditions of successful transfer.

Specimens of tissue were removed by biopsy from papillomas in the skin of adult animals, and, after treatment for ½ hour in a solution of penicillin, fragments were transferred to the brains of other adult animals. Five adult papillomas ranging from 17 days to 3 months in age were tested in this manner, and all proved to be transplantable. The percentage of takes (50 per cent) was less than that noted in the case of papillomas induced in embryonic skin, but the transplants grew to a comparable size and showed the same histological structure (Fig. 10).

A third generation transfer was carried out in one instance, and, although growth was obtained, the incidence of takes was further diminished, and the resulting tumors were smaller than those obtained in the second serial generation.

Heterologous transplantation of papillomas.—The successful transfer of papillomatous tissue to unrelated rabbits suggested that the growths might survive transfer to alien species. Tissue from papillomas in adult skin as well as from growths induced in embryonic transplants was employed, and transfers were made to the brains of mice and guinea pigs.

Takes occurred in the two foreign species, but the mouse proved a better host, exhibiting a higher percentage of takes as well as a more rapid growth rate (Fig. 3). Embryonic skin papillomas and second generation rabbit transplants of such tumors grew more readily on heterologous transfer than did adult skin papillomas, although no difference could be detected on histological examination of the resulting growths (Figs. 11 and 12).

Growth was slow in the guinea pig, and transplants more than 0.5 cm. in diameter on the 30th day were rarely found (Fig. 13). On the contrary, growth in the mouse was sometimes so rapid as to cause death by the tenth day, with tumors occupying approximately half of a cerebral hemisphere.

Serial guinea pig transfer was not attempted, but growth has been obtained in a third mouse generation. However, as in homologous third generation transfers, the percentage of takes was low, and the resulting tumors were comparatively small.

Induction of papillomas in embryonic rabbit skin utilizing cell-free filtrates.—The observation that papillomatous tissue could be transplanted to new hosts supported the possibility that the papillomas found in embryonic skin transplants might result from growth of suspended cells adhering to the embryonic skin during treatment rather than from a conversion of the embryonic epithelium to papillomatous epithelium. In order to test this possibility, cellular suspensions identical with those used in previous experiments were injected directly into the brains of adult rabbits. Suspensions of adult domestic papilloma, papilloma induced in embryonic skin, and glycerinated preparations of cottontail papilloma were used, but no evidence of growth was found a month later. It seemed conceivable, however, that the fragments of embryonic skin might form a more suitable nidus for the suspended papilloma cells than brain tissue and support their growth. Accordingly, pieces of embryonic lung were bathed in the various preparations and transplanted to adult brains. The embryonic lung survived and grew, but no growths

were found that could be related to adherent papilloma cells. Conclusive evidence, however, appeared to depend on a duplication of the results with cell-free filtrates, and pertinent experiments were undertaken.

It was found that filtrates of glycerinated cottontail tissue, fresh papillomas induced in the adult skin of domestic rabbits, and papillomas induced in embryonic skin were all effective and elicited papillomas if applied to embryonic rabbit skin prior to brain transfer (Figs. 14 and 15). The incidence of the lesion and that of complete transformation of the transplant were not so great with filtrate-treated skin as with skin treated with cellular emulsions; but in all of the cases studied (three glycerinated preparations, three adult papillomas, and six embryonic papillomas), one or more of the test transplants contained papillomas. Furthermore, a longer interval of time was required. In most of the transplants examined before the 30th day, the areas of papillomatous growth were small, isolated, and surrounded by normal-appearing skin and could readily be missed unless adequate sections were cut.

The induction of papillomas in embryonic rabbit skin transplanted to alien species.—Persistence of the infectious agent in transplants in mice, together with the known ability of embryonic rabbit skin to survive and grow in the brain tissue of this species, suggested that papillomas might be induced in embryonic rabbit skin transplanted to mouse brain. Accordingly, a series of experiments was undertaken involving the transfer of embryonic rabbit skin treated with suspensions or filtrates of papillomatous tissue to the brains, eyes, and subcutaneous tissues of mice.

Subcutaneous transplants persisted with little growth throughout the experiment, but papillomas were not found on microscopic examinations. On the contrary, transplants to the anterior chamber of the eye and to the brain grew progressively and were rapidly converted to papillomatous tissue. The anterior chamber was often completely replaced by the transplant on the fourteenth day, and sections at that time revealed large areas of papillomatous transformation. Mice bearing brain transplants were sometimes found dead or comatose during the third week, and sections of the brain showed large growths compressing and distorting normal structures. Histologically, the papillomas induced in mice were identical with those obtained in rabbits and, in like manner, survived and grew on serial transfer (Figs. 17 and 18).

In general, filtrates proved less efficacious in inducing papillomas in mice than did cellular suspensions. Tissue derived from papillomas induced in embryonic skin in rabbit brain transplants gave a higher percentage of positive results than glycerinated cottontail material, and fresh adult papillomas were least effective.

Attempts to induce papillomas in treated embryonic rabbit skin transplanted to guinea pig brains were successful in several cases (Fig. 19). The guinea pig, however, is a poor host for embryonic rabbit tissues as compared to the mouse, and similar results could not be expected.

Induction of papillomas in the skin of adult domestic rabbits with material derived from papillomas of embryonic skin.—The papillomas induced in embryonic rabbit skin have been tested routinely to determine their ability to elicit growths in the scarified epidermis of adult domestic rabbits. The significance of the results of this test is questionable for, in a great majority of cases, the animals were released as having negative results at the end of a month. However, several were retained for breeding, and four of these subsequently developed papillomas in the scarified areas.

Three of the animals belonged to one experiment and represented the test of a papilloma induced in embryonic rabbit skin in the brain of an adult rabbit and then transferred as a fragment to the brain of a second adult rabbit. The original infectious material was a suspension of cottontail papilloma. The induced growth was removed 23 days after transplantation of treated embryonic skin, and the rabbit bearing the transplant of this papilloma was killed 26 days after transfer. Thus, an interval of 49 days elapsed between contact with the preparation of cottontail papilloma and scarification of the test rabbits. The growths in the test animals were noted 72 days after scarification, and, because of frequent handling, it is probable that this is a close approximation of the actual date of their appearance. The papillomas were small, discrete, few in number, and at the present time, 1 month after discovery, have grown to large size but remain isolated.

The fourth animal of the category under consideration was one of two retained in the colony after test of a papilloma derived from treated embryonic rabbit skin transplanted to the brain of a mouse. One of the animals remained negative to the test, but at 69 days small, isolated papillomas appeared at the site of scarification in the other. Histological sections of biopsy specimens taken at this time showed a lesion identical with those obtained after treatment with cottontail papilloma material. Unfortunately, the rabbit died of an intercurrent infection a week later, and the course of the growth could not be followed.

It is regrettable that all the test animals were

not held for longer observation, but the incubation period was considerably longer than experience with cottontail preparations suggested, and its extent was not foreseen. Although the positive results are of limited significance from a numerical point of view, they become of consequence when considered in relation to the great difficulty encountered in transferring infection with the Shope papilloma between adult domestic rabbits, and, despite statistical qualifications, the conclusion seems justifiable that the virus remains infectious and "unmasked" after residence in embryonic rabbit skin.

DISCUSSION

Some of the results obtained in the experiments recorded in this paper suggest conclusions contrary to those derived from other work and require further discussion. These relate to the susceptibility of embryonic tissue to the Shope papilloma virus, the homologous and heterologous transplantation of the papilloma, and the induction of the lesion in alien species.

Papillomas were not observed by Rous and Beard (8) in homologous subcutaneous transplants of embryonic rabbit skin kept in contact with the virus *in vitro* for $\frac{1}{2}$–2 hours. In contrast, Fischer and Syverton (2) obtained papillomas in all rabbit embryos injected with the virus between the eighteenth and twentieth days of intra-uterine life. The earlier investigations suggest that embryonic epithelium is less responsive to the virus than adult skin, while the *in vivo* studies indicate at least a comparable degree of susceptibility. In the present experiments, embryonic skin proved to be as responsive to the cottontail virus as adult skin and far more susceptible than adult skin to the virus derived from domestic rabbits. It should be noted, however, that the experimental methods used in studying the reactions of the two tissues are not equivalent, and a possibility exists that the increased susceptibility observed may relate to the conditions of transplantation rather than to the embryonic nature of the tissue. A comparable study of the susceptibility of transplants of adult skin is under way, and the results will be reported in a later paper.

In any case the fact remains that the Shope papilloma virus can be maintained in domestic rabbits by serial passage in embryonic skin. The typical lesion is induced in the transplant, and the technic offers a convenient test for the presence or absence of the virus.

Attempts to transplant papillomatous tissue were made primarily to test the possibility that the papillomas found in growths of embryonic skin might result from proliferation of the suspended cells adhering to the tissue during treatment. Subsequent studies showed that cell-free filtrates were also effective and necessitated the conclusion that infected embryonic cells were themselves the source of the papilloma, but the observation that the papilloma cells derived from either embryonic transplants or adult skin were capable of survival and growth on transfer to normal unrelated animals of the same or different species is of significance from another point of view.

In a large series of transplantation experiments, involving the homologous and heterologous transfer of rabbit and mouse tumors during different developmental stages, survival and growth in unrelated normal animals were never noted until the

FIG. 1.—Brain of domestic rabbit bearing transplant of embryonic rabbit skin treated with a cell-free filtrate of a papilloma derived from the intact skin of an adult domestic rabbit. The cortex overlying the growing transplant has been dissected away. There is a luxuriant growth of hair, and on section papillomatous transformation was limited to scattered areas as in Fig. 6.

FIG. 2A.—Brain of domestic rabbit bearing transplant of embryonic rabbit skin treated with a cellular suspension of a papilloma derived from a cottontail rabbit. On section the transplant showed complete papillomatous transformation, and there was no growth of hair.

Fig. 2B.—The tumor mass occupying the right hemisphere in Figure 2 has been dissected from surrounding brain tissue and is shown in cross-section.

FIG. 3.—Brain of mouse bearing a third-generation heterologous transplant of a papilloma induced in the intact skin of an adult domestic rabbit. The mouse was killed 30 days after transfer. A normal brain obtained from a mouse of the same age is shown for contrast.

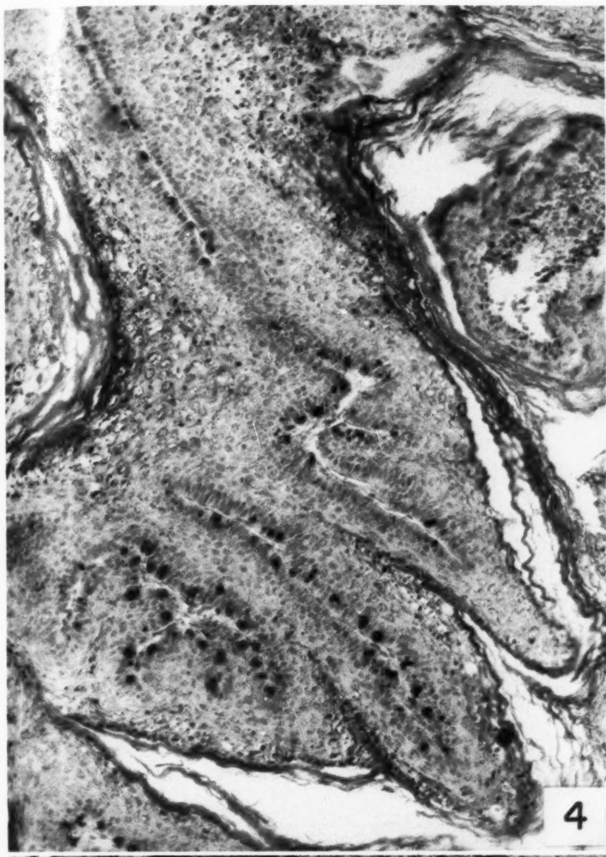

4 5 6 7 8
1
2A
2 16 20 24 28
4 5 6
2B
3 4
3

Fig. 4.—Anterior chamber transplant of embryonic rabbit skin treated with a cellular suspension of a cottontail papilloma. The rabbit was killed 18 days after transfer. ×150.

Fig. 5.—Control transplant of normal untreated embryonic rabbit skin in the brain of an adult rabbit. The animal was killed 25 days after transfer. ×35.

Fig. 6.—Transplant of treated embryonic rabbit skin in the brain of an adult domestic rabbit. Prior to transfer the fragment of skin was bathed for ½ hour in a cellular emulsion of cottontail papilloma tissue, and the rabbit was killed 17 days after transfer. There is an abundance of typical papillomatous tissue, but some normal structures remain. ×45.

Fig. 7.—Brain transplant of embryonic rabbit skin treated with a cellular suspension of a cottontail papilloma. The rabbit was killed 23 days after transfer. All of the epithelium is papillomatous. ×45.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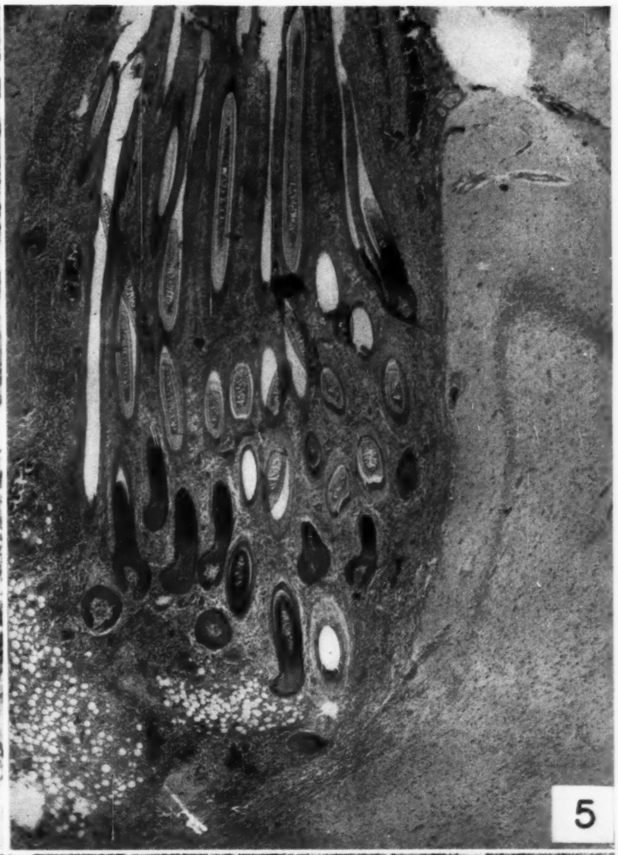
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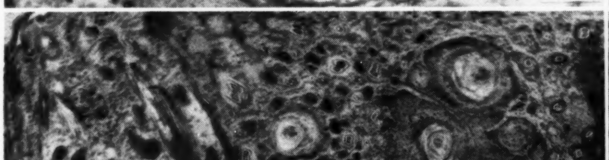
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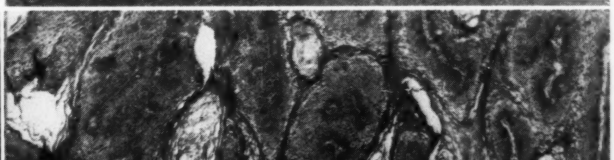
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FIG. 8.—Papilloma induced in brain transplant of embryonic rabbit skin treated with a cellular suspension of the papilloma shown in Figure 7. Fragments of this papilloma (Fig. 7) were first transplanted to the brains of adult domestic rabbits, and one of the growths obtained after 17 days (Fig. 9) was used as the infectious material in the present experiment. ×45.

FIG. 9.—Growth of the papilloma shown in Figure 7, 17 days after transfer to the brain of an adult domestic rabbit. ×45.

FIG. 10.—Growth of a papilloma derived from the intact skin of an adult domestic rabbit, 18 days after transfer to the brain of another domestic rabbit. ×45.

FIG. 11.—Growth of a papilloma induced in a brain transplant of embryonic rabbit skin 22 days after transfer to the brain of a DBA mouse. ×270.

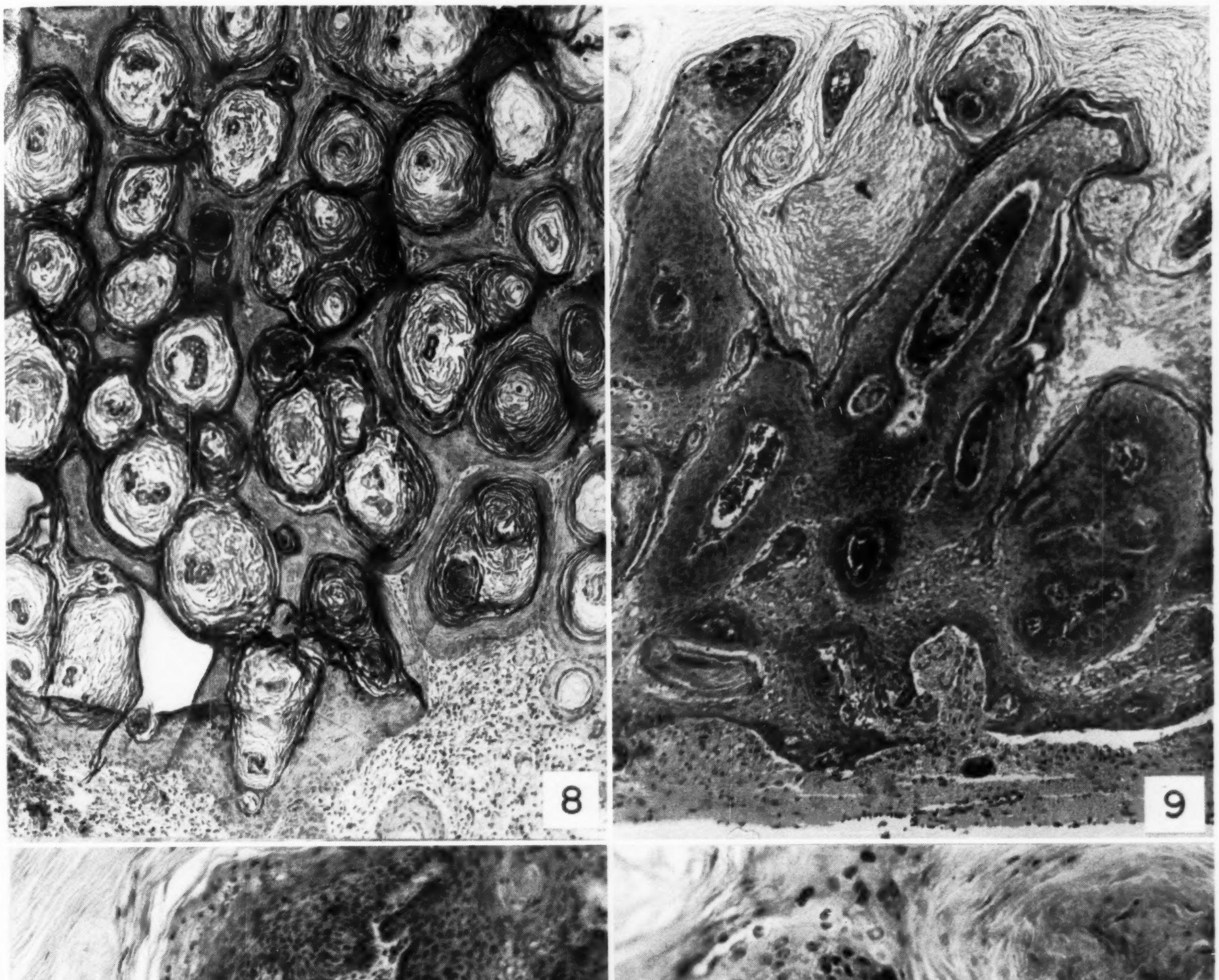
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FIG. 12.—Growth of a papilloma induced in intact adult rabbit skin 23 days after transfer to the brain of a DBA mouse. ×270.

FIG. 13.—Growth of a papilloma induced in a brain transplant of embryonic rabbit skin 30 days after transfer to the brain of a guinea pig. ×150.

FIG. 14.—Brain transplant of embryonic rabbit skin treated with a cell-free filtrate of a cottontail papilloma. Note papillomatous transformation along one segment of periphery with relatively normal appearing skin elsewhere. ×13.

FIG. 15.—Higher-power view of papillomatous area in Figure 14. ×150.

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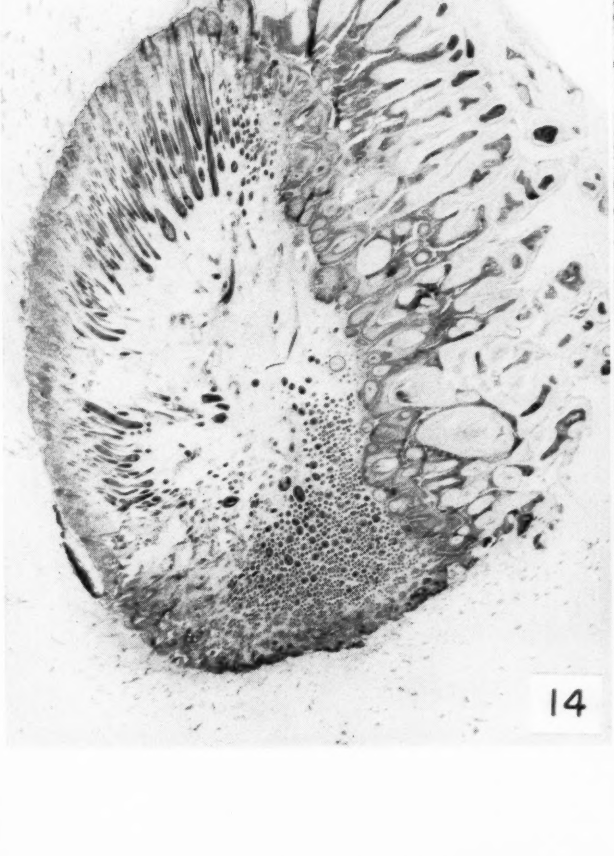

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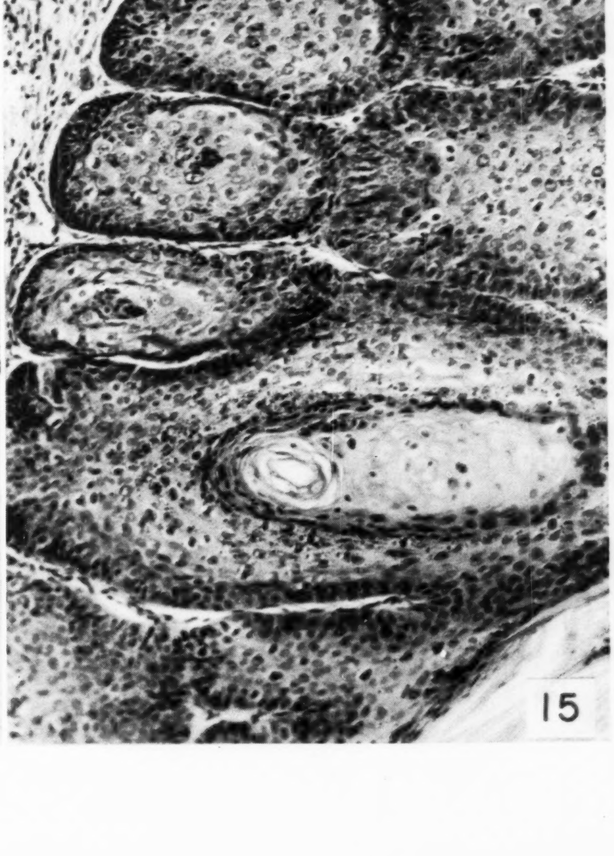

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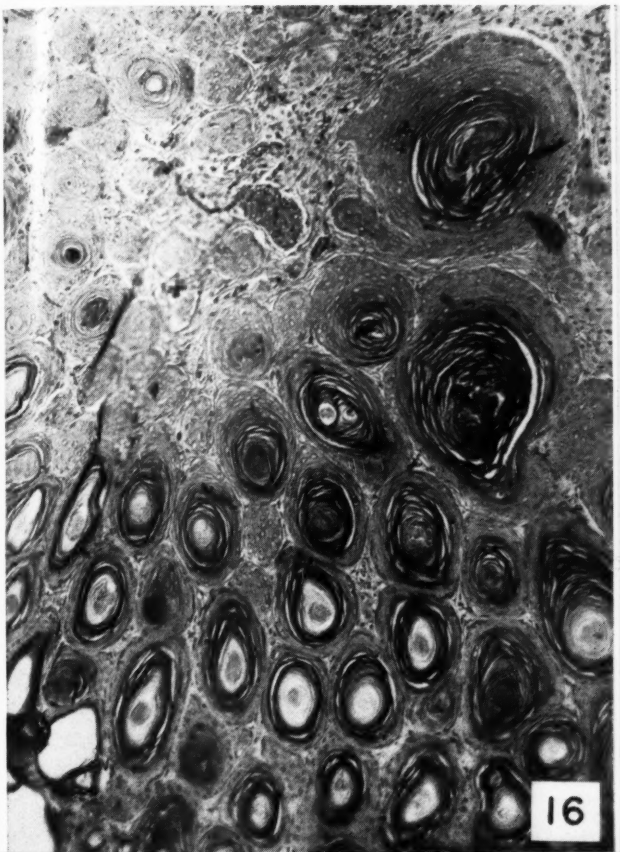
FIG. 16.—Transplant of embryonic rabbit skin treated with a suspension of domestic papilloma. The rabbit bearing the transplant was killed 12 days after transfer. ×120.

FIG. 17.—Transplant of embryonic rabbit skin treated with a suspension of cottontail papilloma 18 days after transfer to the brain of a DBA mouse. Note complete papillomatous transformation of the rabbit skin in a resistant species. ×20.

FIG. 18.—Higher-power view of transplant shown in Figure 17. ×150.

FIG. 19.—Transplant of embryonic rabbit skin treated with a suspension of cottontail papilloma 28 days after transfer to the brain of a guinea pig. ×150.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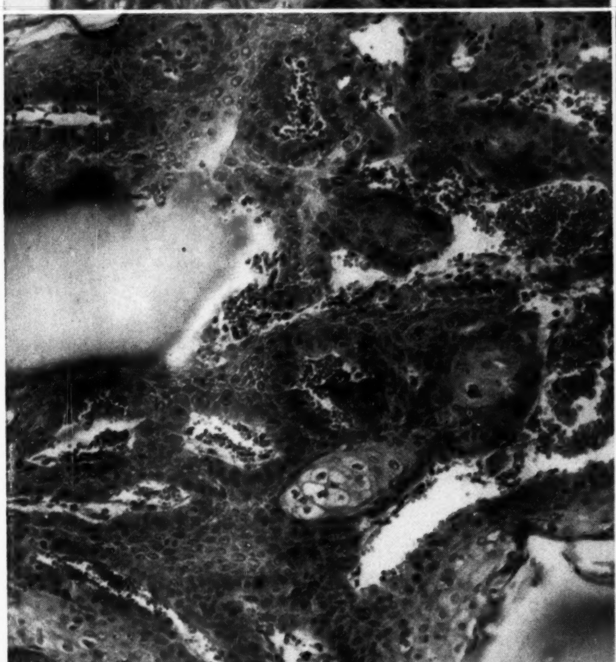
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primary tumor had attained the ability to metastasize (6). Similar results were obtained in studies concerned with the heterologous transplantation of human tumors (7), and it was concluded that metastasizability and the ability to grow in normal unrelated animals or in heterologous species were coincident attainments in tumor development.

The ability of the Shope papilloma to grow on homologous and heterologous transfer during premetastasizable phases of development is shared by the Rous chicken sarcoma (1, 5, 9) but by no other tumor in our experience. It appears significant that the Rous sarcoma, like the Shope papilloma, can be readily transplanted to the brains of animals of alien species at any period after the appearance of tumor in the virus-infected area, and it is suggested that such behavior may be a unique attribute of virus-induced tumors differentiating them from growths of different etiology. An investigation of other tumors in premetastasizable developmental phases suggested that during this phase the tumors were dependent for continued existence on factors peculiar to the tumor-bearing animal, and in as much as the factors were not supplied by normal animals, growth in normal animals did not occur. On such a basis, it must be assumed either that the Shope and Rous tumors have no dependent phase of development, or that the factors on which continued existence depends are carried by the tumors themselves. The latter is, in fact, known to be the case, and, from this point of view, the behavior of the Shope papilloma and the Rous sarcoma is not contrary to that of other tumors but represents, rather, a special circumstance in which the dependent factor is a virus residing in the tumor cells and carried with them to new hosts.

The ability to induce the Shope papilloma in transplants of embryonic rabbit skin in species resistant to the infecting agent offers an approach to a number of problems associated with the behavior of this and other viruses but is also of interest from the standpoint of the status of the transplant in relation to the constitution of the host. Transplanted tissues obtain a stroma and vascular supply from the new host and are pervaded by its blood and tissue fluids. It might be expected, therefore, that the growing cells of the transplant would reflect the constitution of the new host and share its reactions. On the contrary, the reverse holds, and the reactions of the transplant are those of the donor species rather than of the recipient host.

Goodpasture and Anderson (3) have demonstrated that human skin and fetal membranes grafted on the chorioallantois of chick embryos are susceptible to a number of viruses which do not infect chick tissues. It has also been shown in this laboratory that fragments of embryonic chick tissue infected with the Rous sarcoma virus and transplanted to the brains of mice develop the typical Rous sarcoma (1). Furthermore, embryonic chick skin infected with the fowl pox virus and transplanted to mouse brain responds with inclusion body formation and an inflammatory reaction identical with that observed in the chicken (1). The point to be stressed here is that the Rous sarcoma virus and the fowl pox virus, like the Shope papilloma virus, are not infectious in the mouse and produce no lesions in the mouse brain. It would appear from these observations either that susceptibility to infection is a function of local tissue factors rather than of the constitution of the host, or that transplants retain the constitutional identity of the donor and thus represent regions of independent reactivity capable of responses foreign to and beyond the capacities of analogous resident tissues despite a common host and immunological environment.

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The Transplantation of Tissues between Zoological Classes*

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The ability of embryonic tissues and of metastasizable tumors of mammalian origin to grow on heterologous transplantation has been demonstrated (2, 3). The investigations concerned were limited largely to transfers between mammalian species, and, although several chicken tumors were found to survive transplantation to the eyes of guinea pigs, mice, and rabbits (11), no detailed exploration of the transplantation reactions between animals of different zoological class was undertaken. Studies by other workers have shown that a variety of mammalian tumors can be successfully transferred to the chorioallantoic membrane (9), the yolk sac (12), and the vascular system (1) of developing chicks and that several mouse sarcomas can be grown in the somatic tissues of chick embryos (8). Such experiments, together with the reciprocal transfer of chicken tumors to mammals, suggested that zoological class distinctions might offer no more effective a barrier to the transplantation of autonomous tissues than is effected by species differences. Accordingly, a series of pertinent transplantation studies was instituted, and the present paper reports the results of exploratory experiments.

In general, the experiments concerned the transplantation of embryonic and cancer tissues between the rabbit, guinea pig, or mouse on the one hand and the chicken and chick embryo on the other. Their purpose was primarily to study transplantability, but in several instances an attempt was made to determine the constitutional status of the transplants with particular reference to susceptibility to various infectious agents.

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MATERIALS AND METHODS

The methods of anterior chamber and brain transplantation in mammals have been previously described (3, 5). Similar technics were used for hatched chickens. The transplantation site of the chick embryo was the mesencephalon of 4-day-old chicks (Hamburger and Hamilton [7], stages 19–24). At these stages, the mesencephalon is the largest and most readily accessible portion of the nervous system, and its large cavity makes an excellent chamber in which to place tissue fragments. After carefully removing a window in the egg shell and shell membrane, the remaining procedures were carried out under a binocular dissecting microscope. The amnion was grasped gently with a pair of fine watchmaker's forceps, and a slit was made in the membrane by means of a glass needle. The same needle was used to make a hole in the lateral wall of the mesencephalon. If, through rough manipulation, the amnion became extensively torn, the subsequent procedures of incising the mesencephalic wall and implanting the fragment were made considerably more difficult, since holding the amnion with the forceps provides stability. The tumor fragments usually measured 0.5 mm. or less in diameter, were transferred to the incision by a small glass pipette, and were pushed through the wall with the operating needle. The edges of the amnion were approximated, a piece of cellophane tape was placed over the shell defect, and the egg was returned to the incubator. After operation the eggs were not turned. The hazards of this operation are numerous, and the operative mortality was high. The membranes and blood vessels at this stage are very delicate and easily broken. The nervous system is especially well vascularized, and the incision of necessity results in more or less hemorrhage.

RESULTS

Tumor Transplants

Mammals to chicken.—The mammalian tumors used for transfer to chickens consisted of the Brown-Pearce rabbit carcinoma and the mouse neuroblastoma, C1300.

The results of transplantation of the Brown-Pearce tumor to hatched chickens varied with the age of the bird at the time of transfer. When 1-day-old chicks were used, transfer was successful, and tumor cells persisted until the 22d day. The growth was easily identifiable on cross section of the brain, and on microscopic examination masses of cells identical to those found in homologous transplants of the tumor (Fig. 5) could be observed. The transplants were almost invariably surrounded and infiltrated by small mononuclear cells,

presumably lymphocytes. Such reactive cells were present on the 5th day and increased in number with age of the transplant. In a few instances the tumor was found in Virchow-Robin spaces (Fig. 6), and one tumor devoid of cellular reaction was found in an animal 32 days after transfer (Fig.7).

Transfer of the tumor to the brains of adult chickens was apparently unsuccessful, and no cells identifiable as derived from the transplanted fragment could be found at the end of a week. The site of transplantation, on the contrary, was the seat of a dense accumulation of lymphocytes.

A more detailed study of the age periods between hatching and adulthood is in progress, but present data, consisting of random samples, are sufficient to indicate the significance of age as a primary factor in the lymphocytic response and the success of transplantation.

Transfer of the Brown-Pearce tumor to chick embryos was carried out on the fourth day of incubation, and the embryos were examined and removed for histological study at various intervals after operation. None of the animals which had been operated on were allowed to hatch, for it was felt that the large tumer herniating through the cranium would interfere with the hatching process.

Embryos studied on the fourth day after transfer bore small growing tumors made up of typical Brown-Pearce cells with many mitotic figures. Vascularization was established, and the lymphocytic reaction, characteristic of the hatched chicken, was absent (Fig.1). Two weeks after transfer, i.e., on the 18th day of incubation, the growth had reached a large size (occupying the equivalent of half of a cerebral hemisphere and frequently measuring as much as 8 mm. in diameter) and presented the characteristic appearance of the tumor when grown in the brain of the rabbit. Microscopically, there was excellent vascularization and less necrosis or reaction than is generally found in the tumor in its natural host.

Growth in the embryonic mesencephalon appeared to be expansive rather than invasive in character, and the expanding mass was incorporated into the developing brain structure. This is well illustrated in Figure 2, where the ependymal cells covering the tumor have not been modified and appear entirely similar to those lining the uninvolved ventricle. It should be emphasized that the lymphocytic response, observed after hatching, was never found in the embryonic brain.

The results obtained on transfer of the mouse neuroblastoma, C1300, were similar. Good growth followed transplantation to the embryonic mesencephalon (Fig. 4), while a lymphocytic reaction characterized the response of hatched chicks.

Chicken to mammals.—The material utilized in attempts to transfer chicken tumors to the brains of mammals was limited to the Rous sarcoma. This tumor, or the variant carried in the duck, was found to grow readily in mice, guinea pigs, and rabbits and to survive serial passage in these species.

Transfer to the heterologous hosts could be effected as soon as the tumor in the chicken or duck became palpable, and the percentage of takes was not modified by continued residence of the tumor in the parent species. Furthermore, the manner of induction of the tumor in the primary host, either by cell-free filtrates or tumor fragments, did not influence the heterotransplantability of the resultant growth.

Growth in the brain was rapid and often resulted in a tumor mass which occupied half of a cerebral hemisphere by the 10th or 15th day. Animals killed at later periods bore regressing growths, but intact tumor cells, demonstrated by transfer to be viable, were found as late as the 30th day. Mice frequently died with neurological signs between the 7th and 11th days, but rabbits and guinea pigs rarely gave evidence of increased intracranial pressure.

At autopsy the inoculated hemisphere was swollen, and on section the typically viscid, semitranslucent tumor was easily distinguished from surrounding brain substance. Histologically, the tumor was identical in structure and cellular composition with the tissue obtained from the parent species (Figs. 8 and 9). The surrounding brain was not compressed, and growth appeared to be invasive rather than expansive in character. Except in the presence of extensive necrosis, the adjacent brain was free of lymphocytic or other cellular reaction despite the heterologous nature of the transplanted tumor.

Serial transfer was readily effected, and there was no diminution in the incidence of takes or growth rate after seven consecutive passages in the three different species. Transfer back to the chicken or duck was attempted at various times during serial passage, and growth was always obtained.

Transfer of tumor tissue to the intermuscular and subcutaneous spaces of alien hosts was attempted with material derived from brain growths, but the results were not different from those following the transplantation of tissue obtained directly from the chicken. In such cases, an immediate inflammatory reaction destroyed the tumor cells and resulted in an exuberant mass of granulation tissue which, unless sectioned, might

be mistaken for growth. Intact tumor cells persisted for longer periods of time in the subcutaneous tissues of DBA mice than in corresponding sites in C3H and other mouse strains. It is of considerable interest that progressive growth of subcutaneous transplants was observed in several experiments involving the use of C3H mice bearing spontaneous mammary tumors. In all such cases the primary mammary tumor had metastasized, whereas no growth of the transplanted Rous sarcoma was obtained in other tumor-bearing C3H mice whose tumors had not metastasized.

In several experiments attempts were made to transplant the Rous sarcoma grown in mouse brain to the subcutaneous spaces of other mice in association with fragments of embryonic organs, for it had been noted previously (4) that the Brown-Pearce rabbit tumor could be grown in the subcutaneous space of refractory mouse strains in this manner. Growth of the Rous sarcoma was obtained in fragments of embryonic mouse tissue transplanted to C3H mice but remained confined to the growing transplant and did not extend into host tissues. In such cases, the tumor rapidly lost its myxoid character, and the cells came to resemble those of adult fibrous connective tissue. The resemblance of the tumor to mature granulation tissue of host origin was marked, and its identity as the Rous sarcoma could only be established by the occurrence of typical histological features after retransfer back to the chicken.

It was also noted that the Rous sarcoma derived from alien brain transplants, as well as tissue obtained directly from the chicken, grew well in association with transplants of other tumors. An illustration of such growth is shown in Figure 10. In this instance the Rous sarcoma and the mouse neuroblastoma, C1300, were transplanted simultaneously to the anterior chamber of a guinea pig's eye. The Rous sarcoma grows to a larger size and persists for a longer time under such conditions than when transplanted alone.

Embryonic Tissue Transplants

The transfer of mammalian embryonic tissues to the brain or eye of the chicken is associated with a pronounced lymphocytic reaction, and, although some growth of the transplant is apparent, its extent and duration are largely obscured. It should be noted that a comparable reaction follows the homologous transplantation of embryonic tissue in the chicken and thus appears to relate to a peculiarity of the host rather than to the constitution of the transplant. In contrast, transplants of chicken embryonic tissue to the eye and brain of the rabbit, guinea pig, and mouse do not induce an inflammatory reaction beyond vascularization and offer a unique material for experimentation.

Growth characteristics.—A variety of embryonic chicken tissues have been successfully transplanted in mammals. The growth characteristics of the different tissues are essentially the same. Takes are apparent in the eye and brain within 5 days of transfer, and their early behavior does not distinguish them from homologous embryonic transplants. Maximum growth is usually attained by the third week, and, although regression occasionally occurs, the majority of transplants persist. Histological study shows differentiation and organization into the adult counterparts of the embryonic tissues used, and in some instances there is evidence of function such as the production of keratin by squamous epithelium and of mucus by intestinal epithelium (Figs. 11–16).

Constitutional status.—The ability of embryonic chick tissues to grow and differentiate into mature, adult organs without inducing a foreign-body reaction in mammalian hosts suggested a constitutional status different from that attained during the course of normal development, and experiments were instituted in an attempt to explore this suggestion. The approach was based on the differential susceptibility of mouse and chicken tissue to the Rous sarcoma virus and the virus of fowl pox and consisted of a study of the reactions of infected embryonic chicken tissue transplanted to mouse brains. It should be noted that the mouse is refractory to infection with these agents, whereas characteristic lesions develop in the chicken.

Fragments of mesodermal tissue, cartilage or muscle, derived from a 10-day-old chick embryo, were bathed in cell-free filtrates of the Rous sarcoma and then transplanted to the brains of adult mice. The mice were killed 10–17 days after transfer, and the brains were sectioned through the site of inoculation. The results varied with the filtrate used. In some instances, the transplants appeared normal in all respects and showed no evidence of infection with the Rous virus. In the case of other filtrates, however, the majority of transplants contained foci of proliferation made up of cells identical in structure and arrangement with those found in the typical Rous sarcoma (Figs. 17 and 18). The usual nidus of induction of the sarcoma was in the perichondrium of embryonic cartilage, as shown in the illustrations, but foci were also found in transplants of muscle and connective tissue.

Similar experiments were carried out utilizing

embryonic chicken skin bathed in cell-free filtrates of fowl pox tissue before transfer to mouse brains. The results were consistent, and in all cases fowl pox lesions were present in the transplants (Figs. 19 and 20).

DISCUSSION

The experiments described in this paper were undertaken as part of an attempt to define the limits of heterotransplantability, and the results demonstrate that the differences between zoological classes, as represented by the chicken on the one hand and the rabbit, guinea pig, and mouse on the other, do not constitute effective barriers to the transplantation of cancer and embryonic tissue. Transplants between zoological classes are representative of a greater divergence in tissue and humoral relationships than is associated with interspecies transplants and are thus more significantly indicative of the autonomous nature of the tissues in question. The autonomy or independence of biochemical immunological environment, demonstrated by the ability of a tissue derived from a rabbit to survive and grow in a chicken and vice versa, would appear to be a reflection of a constitution characterized by a lack of specificity and a high degree of self-sufficiency and, in these respects, offer a sharp differentiation from normal, adult states.

The transfer of mammalian embryonic tissue to the brains of adult chickens met with only limited success, for, although there appeared to be some growth, the accompanying mononuclear cell reaction was so marked as to obscure its extent and character. A similar situation obtained with respect to transplants of the Brown-Pearce carcinoma, although this tumor grew well and evoked no cellular response in the brain of the developing chick embryo. Murphy has suggested that at some time between the 18th day of incubation and hatching the chick develops an ability to combat and destroy foreign transplants (10). It should be emphasized, however, that the refractoriness is not limited to foreign tissues but includes homologous transplants as well. In fact, the transplantation of embryonic chick tissue to the brain, eye, or muscle of the adult bird is followed by a more intense lymphocytic response than characterizes the transfer of heterologous tissue, and frequently such reactions are so marked as to stimulate a lymphosarcoma in microscopic appearance. It would appear from such observations that the lymphocytic response to transplants of mammalian embryonic and tumor tissue is not concerned with their heterologous nature, but, on the other hand, represents an expression of a constitutional character of the chicken. This characteristic is not expressed in the embryo but is apparent at the time of hatching and develops with age.

In contrast, embryonic chick tissues may be transplanted with relative ease to the brains of adult mammals. Furthermore, the Rous chicken sarcoma grows readily in an adult mammalian environment and may be passed serially in a variety of species. Such transplants represent the reciprocal of those employing the adult chicken as a host, and the absence of a cellular or foreign-body reaction lends support to the suggestion that this response reflects the constitution of the host rather than that of the transplant.

The development of embryonic chick tissues continues in the mammalian environment, and differentiation and organization proceed to the end result of identifiable adult structures. The inference of such behavior is that the cells of the tissue used for transfer are sufficiently independent of their environment that the already initiated processes of differentiation and organization may proceed outside their natural habitat, a suggestion implied by the numerous publications on the *in vitro* cultivation of these tissues in heterologous media.

It seems reasonable to assume that if morphological maturation of a transplant of embryonic chick tissue were accompanied by the development of other biological features typical of adult chicken tissue, the attainment of such character would lead to the same foreign-body reaction in the mouse that distinguishes the transfer of adult chicken tissue. However, such a reaction does not occur, and a question arises concerning the constitutional status of the transplant and its relationship to adult chicken tissue. An attempt was made to supply pertinent data by determining the reaction of the transplant to several viruses specific for chicken tissue but noninfectious to mouse tissue. It was found that the specific disease developed in the mouse-borne transplant, demonstrating that the response characteristic of the chicken was retained, and that with respect to this factor the tissue was chicken in identity. It would appear, therefore, that the immunological factors concerned in the induction of a foreign-body reaction are distinct and separable from those concerned in response to infection. In other words, the transplants are chicken in nature relative to susceptibility and not chicken in relation to antigenicity.

A similar paradoxical situation obtains with respect to embryonic rabbit skin transplanted to the mouse brain. The foreign-body reaction char-

acteristic of alien adult tissue is not induced, and yet this tissue retains its susceptibility to the Shope papilloma virus (6). These observations warrant further inquiry from the viewpoints of biology and ecology, but whatever their interpretation the fact remains that a disease can be induced in embryonic tissue of a susceptible species transplanted into a refractory species, and the technic offers a fresh approach to a number of problems in the field of immunology.

The ability of the Rous sarcoma to survive transplantation to the subcutaneous space of C3H mice bearing metastasized spontaneous tumors in contrast to its failure to grow in normal mice or in mice bearing tumors in earlier stages of development requires emphasis from a different standpoint and constitutes further evidence that an altered constitutional status is associated with metastasis. Comparable behavior has been noted in the Brown-Pearce rabbit tumor, and an enhancement of growth rate, invasibility, and metastasizability has been found to characterize the course of transplanted mouse tumors in C3H mice during the autonomous phase of spontaneous cancer (4). The nature of the constitutional change and of the factors concerned in the increased growth of the Rous sarcoma when transplanted to the guinea pig's eye in conjunction with another heterologous tumor are the subjects of continued investigation.

SUMMARY

The results of heterologous transplantation of tumors and embryonic tissues between zoological classes are described. The Brown-Pearce rabbit carcinoma and the mouse neuroblastoma, C1300, grew very well in the brains of 4-day embryo chicks but, when transplanted to the brains of adult chickens, resulted in small growths eventually destroyed by intense lymphocytic reactions. Embryonic tissue, both homologous and heterologous, elicited a similar but more pronounced reaction when transplanted to the brains of adult chickens. The Rous sarcoma grew well when transplanted to the eyes and brains of mice, guinea pigs, and rabbits. Embryonic chicken tissue transplanted to the eyes and brains of mammals grew and differentiated without eliciting a foreign-body reaction on the part of the host. Although lacking this immunological characteristic of adult chicken tissue, they maintained their susceptibility to the virus agents of the Rous sarcoma and fowl pox by developing characteristic lesions in the transplantation site.

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Fig. 1.—Brown-Pearce tumor growing in the ventricle 4 days after transfer to the brain of a 4-day chick embryo. ×100.

Fig. 2.—Ependyma covering Brown-Pearce tumor in chick embryo brain. ×500.

Fig. 3.—Extent of Brown-Pearce tumor 14 days after transfer to 4-day chick embryo brain. ×12.

Fig. 4.—Appearance of C1300 16 days after transfer to the brain of a 4-day chick embryo. ×500.

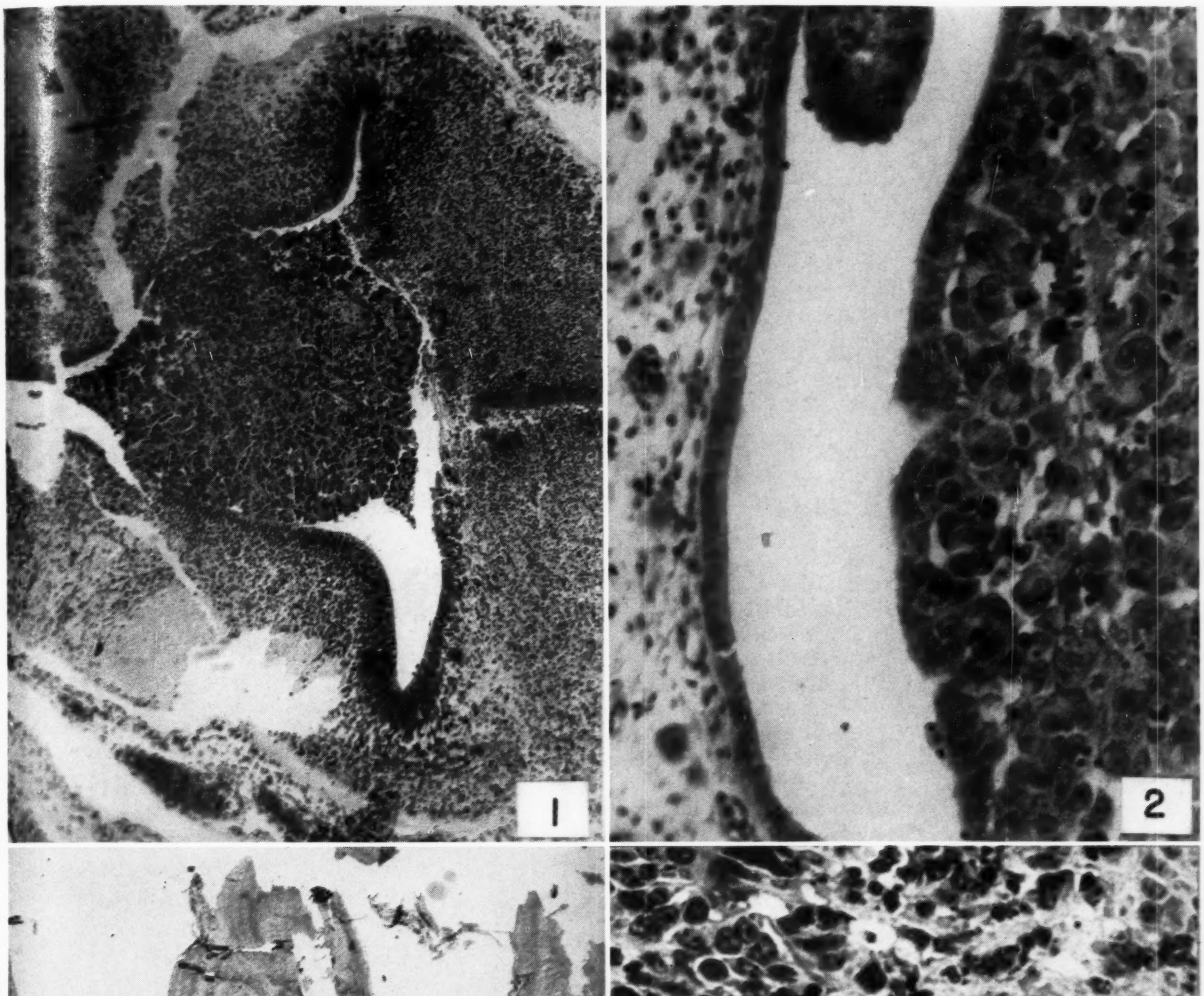
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FIG. 5.—Brown-Pearce tumor transferred to brain of a 1-day hatched chicken. Animal killed 22 days later. Note extensive infiltration of tumor by lymphocytes. ×240.

FIG. 6.—Brown-Pearce tumor 14 days after transfer to brain of a 1-day hatched chicken. Note invasion of Virchow-Robin space by tumor (upper right corner). ×240.

FIG. 7.—Brown-Pearce tumor 32 days after transfer to the brain of a 26-day-old chicken. Identifiable tumor present without cellular reaction. ×300.

FIG. 8.—Transplant of Rous chicken sarcoma in the brain of a rabbit killed 11 days after transfer. ×240.

FIG. 9.—Second generation transplant of Rous chicken sarcoma in the brain of a mouse killed 7 days after transfer. Note the absence of a cellular reaction to the heterologous tumor in the brain tissue in this and the preceding figures. ×240.

FIG. 10.—Coincident transplant of the Rous chicken sarcoma and the mouse neuroblastoma, C1300, to the anterior chamber of a guinea pig's eye. ×200.

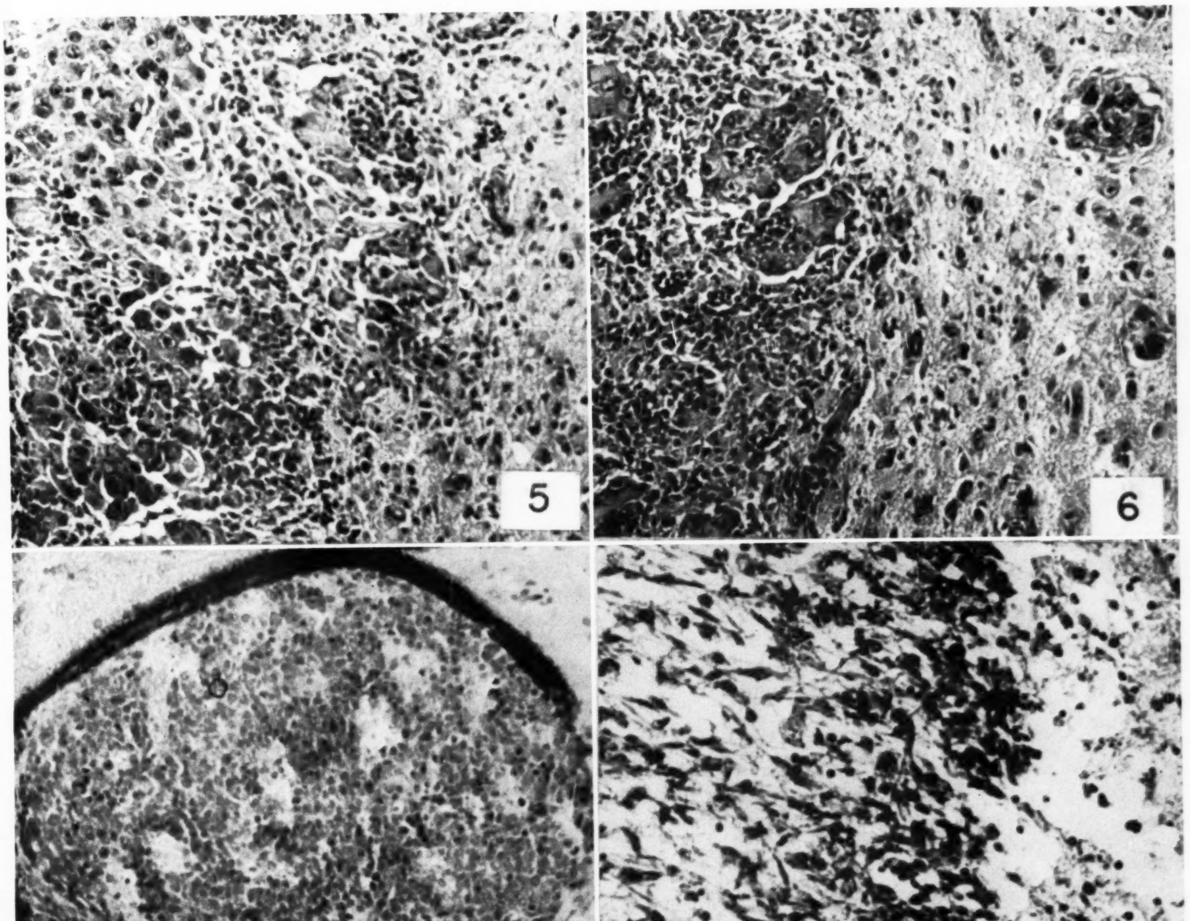
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FIG. 11.—Transplant of heart muscle from a 10-day-old chick embryo to the anterior chamber of a guinea pig's eye. The guinea pig was killed 25 days after transfer. ×180.

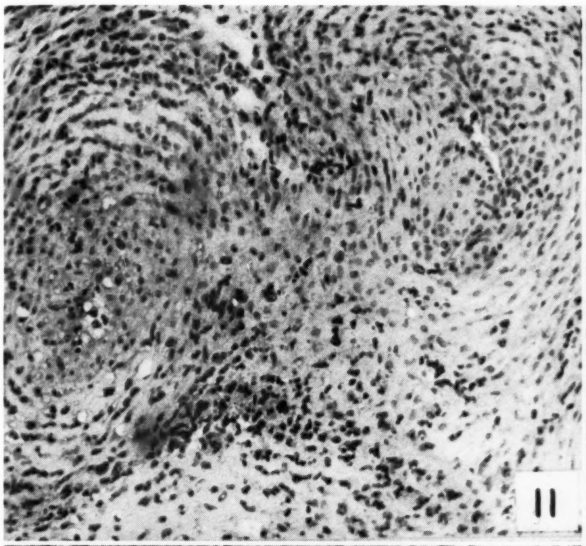
FIG. 12.—Transplant of muscle from the gizzard of a 10-day-old chick embryo to the anterior chamber of a guinea pig's eye. The guinea pig was killed 30 days after transfer. ×240.

FIG. 13.—Transplant of skin from a 10-day-old chick embryo to the brain of a DBA mouse. The mouse was killed 15 days after transfer. ×160.

FIG. 14.—Transplant of kidney from an 8-day-old chick embryo to the anterior chamber of a guinea pig's eye. The guinea pig was killed 12 days after transfer. ×600.

FIG. 15.—Transplant of testicle from a 10-day-old chick embryo to the anterior chamber of a guinea pig's eye. The guinea pig was killed 110 days after transfer. ×340.

FIG. 16.—Transplant of intestine from an 8-day-old chick embryo to the brain of a DBA mouse. The mouse was killed 25 days after transfer. ×550.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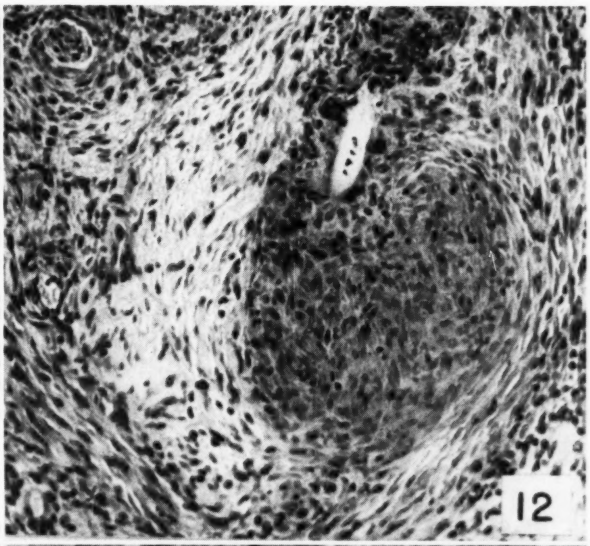
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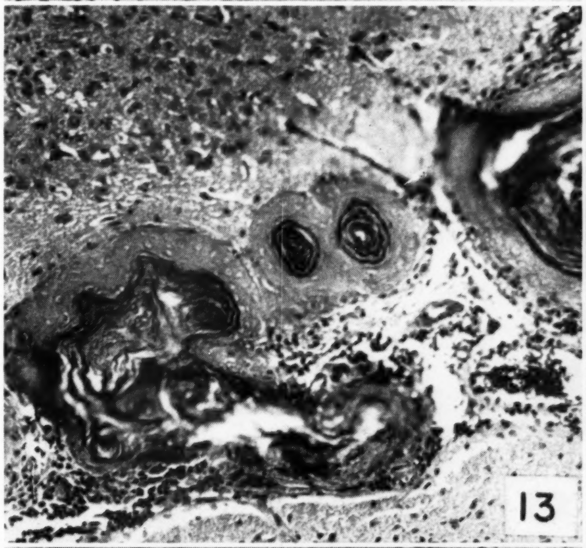
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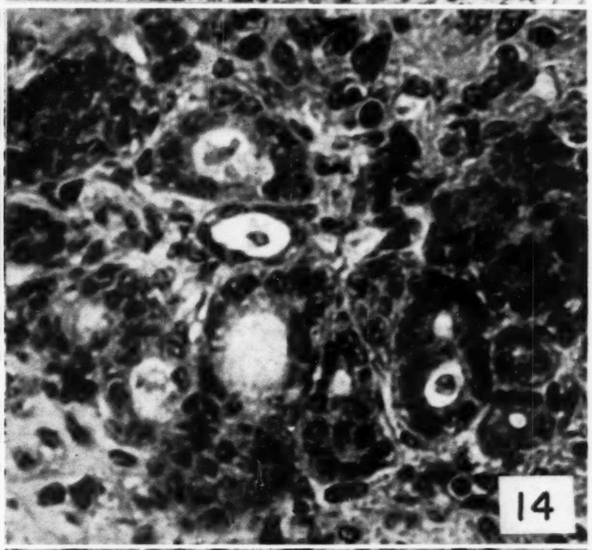
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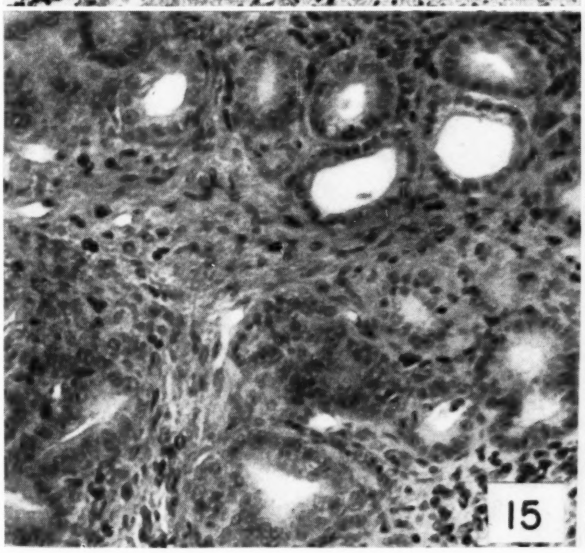
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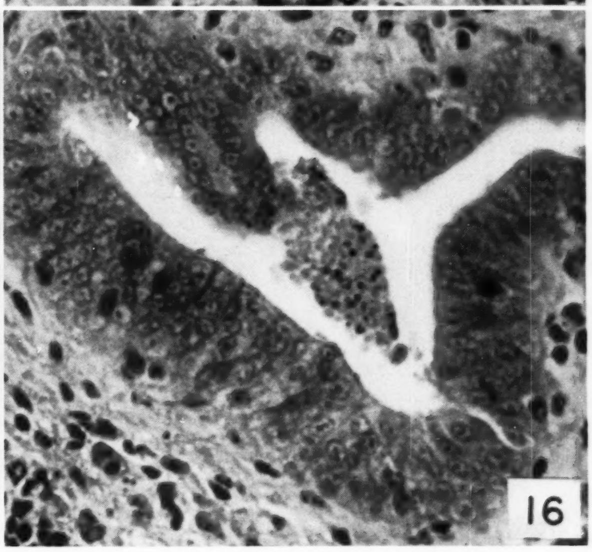
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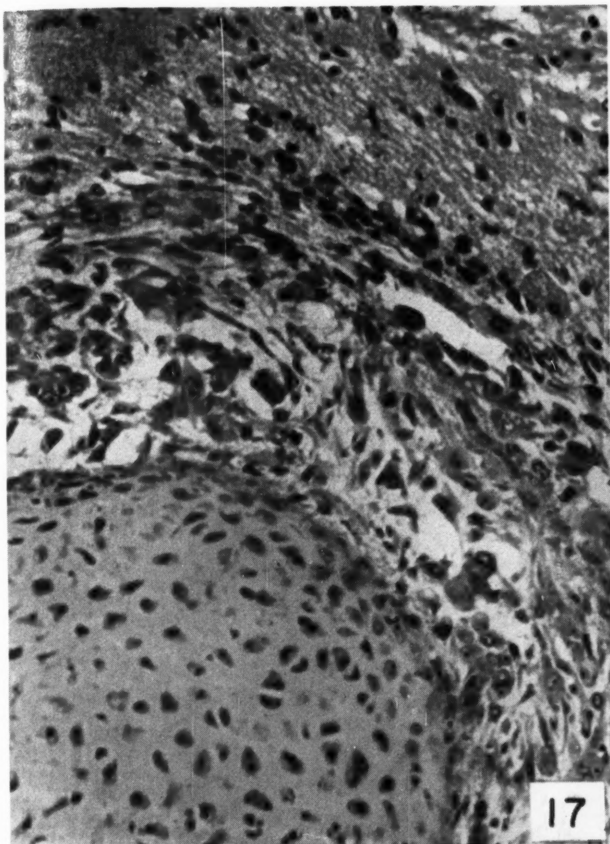
FIG. 17.—Transplant of a fragment of tissue from an 8-day-old chick embryo to the brain of a DBA mouse. Prior to transfer the fragment was bathed in a cell-free filtrate of the Rous chicken sarcoma. The mouse was killed 12 days after transfer. Note the Rous sarcoma arising in the perichondrium of the transplanted cartilage. ×330.

FIG. 18.—Transplanted fragment of 8-day embryonic chick tissue treated similarly (Fig. 17). The mouse was killed 15 days after transfer. Note again the typical Rous sarcoma arising in the transplant. ×330.

FIG. 19.—Transplant of skin from a 10-day-old chick embryo to the brain of a DBA mouse. Prior to transfer the skin was bathed in a cell-free filtrate of a fowl pox lesion. The mouse was killed 10 days after transfer. Note the typical inflammatory reaction in the included dermis. ×400.

Fig. 20.—High-power view of transplant shown in Figure 19. Note masses of intracellular inclusion bodies. ×1,000.



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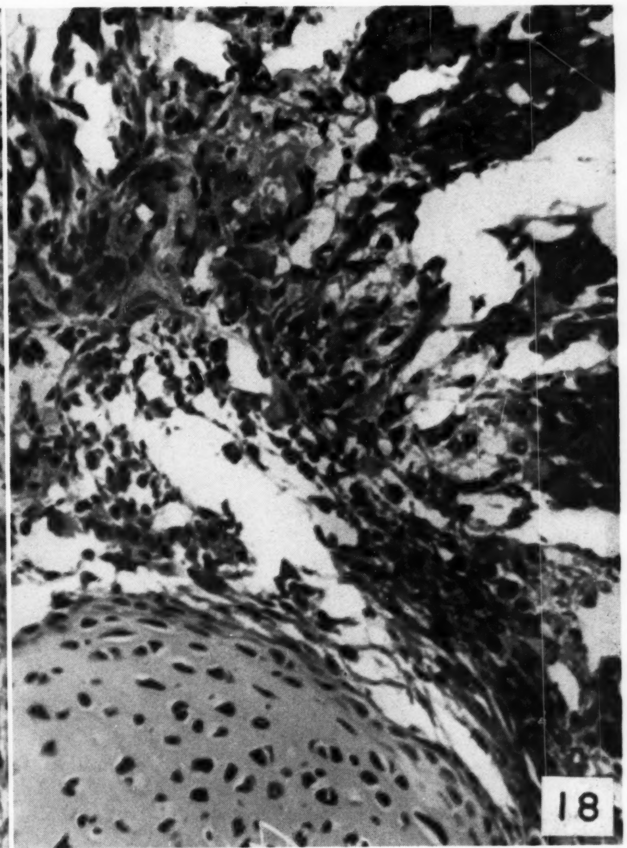

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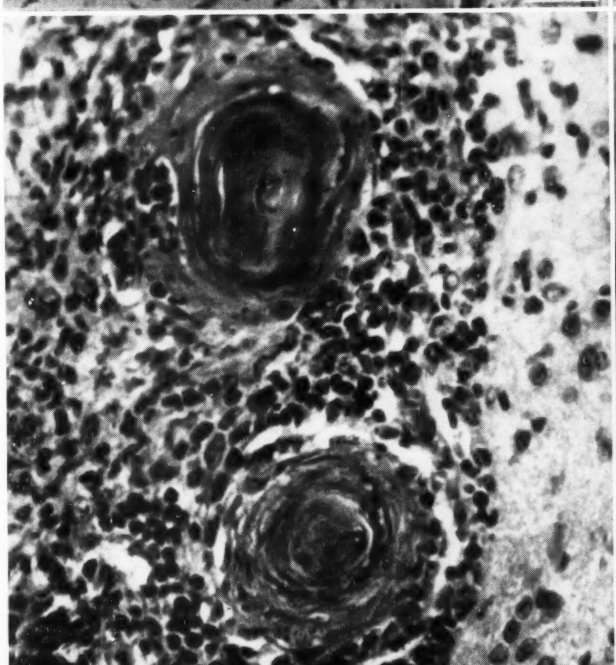

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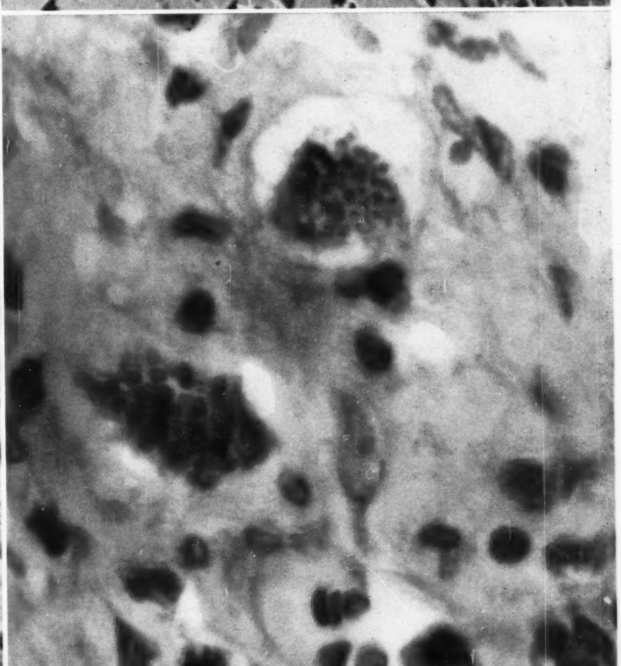

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Effect of Light-activated Benzpyrene on Urease Activity*

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For a number of years the view has been widely held that sulfur compounds are concerned in some manner with the production of tumors by aromatic carcinogens. The best known effect of simple polycyclic hydrocarbons upon sulfur metabolism is the formation of mercapturic acids. Since previous investigations in this laboratory (8, 9) have shown that mercapturic acids are not present in the urine after ingestion of benzpyrene, an interference of the carcinogenic hydrocarbons with sulfur metabolism must involve some other mechanism.

The dependence of a number of enzymes on free sulfhydryl groups for their activity has led to the supposition that the carcinogenic hydrocarbons might react with sulfhydryl groups of one or more enzymes controlling the growth processes. If such a reaction were of a strictly chemical nature, it should be demonstrable *in vitro* with all protein sulfhydryl groups, provided that the latter are on an exposed surface of the protein molecule. Rondoni and Barbieri (18), Rondoni and Bassi (19), and Gaetani (7), using cathepsin and papain preparations, have presented results which indicate that carcinogenic hydrocarbons will inhibit the action of these "sulfhydryl"-dependent enzymes. On the other hand, the results obtained by Feigenbaum (6) do not support the conclusions of the Italian investigators. Mueller and Rusch (16) found no inhibition of a commercial urease preparation by benzpyrene unless the latter had been irradiated previously. Following irradiation of the benzpyrene, inhibition of the urease activity was found to be of two types. The first, which was eliminated by catalase, was likely due to hydrogen peroxide. The second type, which was observed only after a period of prolonged irradiation, was due to some photochemical reaction product of the benzpyrene. In view of the variety of reports on the relation of carcinogenic hydrocarbons to the activity of such enzymes, it seemed advisable to carry out further investigations using a purified enzyme preparation. Urease was chosen because the essentiality of sulfhydryl groups to its activity is well established (10), and it is readily prepared in a purified state. In order to eliminate the possibility of inhibition of the urease by hydrogen peroxide, catalase was added to the experimental samples. Furthermore, all the incubations were carried out in the dark to minimize the effects of light on the benzpyrene.

MATERIALS AND METHODS

Urease was prepared by the method of Sumner (20) and purified by the crystallization procedure of Dounce (5). The urease, when assayed by the method of Sumner, had an activity of 86,000 units/gm of protein. The sulfhydryl content of the urease was determined by a micro amperometric titration as described by Benesch and Benesch (2). The sulfhydryl content of the active urease was 1.7 M/21,000 gm of urease protein.[1] The urease stock solution contained 0.59 mg of nitrogen/ml and showed no appreciable diminution of activity when kept at 2° C. for 3 months. The urease solutions used in the experiments described below were prepared by diluting the stock solution with water. These diluted solutions were allowed to stand 4 hours at room temperature before use, so that the maximum urease activity would be reached (17). Such solutions maintained their activity for several days when stored in a refrigerator.

Commercial crystalline catalase preparation (Delta Chemical Works) was used as an 8 mg. per cent aqueous solution. When this catalase solution was incubated with 5×10^{-4} M H_2O_2 under the conditions used in the urease incubation experiments, the H_2O_2 was destroyed within 6 minutes. A starch-iodide test was used to denote the presence or absence of the H_2O_2. With the aid of a photoelectric colorimeter, the blue starch-iodine color could be detected when the H_2O_2 concentration was as low as 5×10^{-6} M.

Two crystalline samples of 3,4-benzpyrene were used in these studies. The first, Sample A, which was used in Experiment 3, Table 1, was a laboratory preparation which melted at 177° C. (corr.). The ultraviolet absorption spectrum of this benzpyrene sample in ethanol showed all the peaks and points of inflection that are given in the literature (11, 13), with no indication of the presence of impurities. Paper chromatograms of this benzpyrene in caffeine solution, with 50 per cent ethanol as a developing solvent, gave one fluorescent spot with an R_F of 0.92. The second benzpyrene sample, Sample B (Edcan Laboratories), which was used in all the other experiments, was a crystalline preparation melting at 179° C. (corr.). Both of these benzpyrene samples had been stored in colorless glass

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[1] Calculated on the basis of 130,000 units of urease activity per gram of urease protein, which is the value used by Hellerman, Chinard, and Deitz (10).

bottles in a laboratory illuminated with fluorescent lights and with diffuse daylight prior to their use in the initial experiments.

Benzpyrene was dissolved in an aqueous 2 or 3 per cent caffeine solution at room temperature. The undissolved benzpyrene was removed by filtration, and the benzpyrene concentration in the filtrates was determined with a Coleman Photofluorometer. The maximum benzpyrene concentration used was 7.8 mg/100 ml. Both the benzpyrene and the caffeine solutions were prepared and stored at room temperature in the dark to avoid the effect of light upon the compounds (3, 16).

p-Chloromercuribenzoic acid (Sigma Chemical Co.) was dissolved in the minimal amount of 0.1 N NaOH and diluted to a concentration of 1 mg/liter. The 0.01 M cysteine solution was prepared from cysteine hydrochloride and contained an equivalent amount of Na_2CO_3 which was added to neutralize the HCl.

Triple-distilled water was used for all solutions. All glassware was cleaned with concentrated HNO_3 to remove traces of urease inhibitors.

Since hydrogen peroxide developed in solutions of benzpyrene has been shown to inhibit urease (16), every precaution was taken to eliminate hydrogen peroxide in the incubated samples. Consequently, the procedure which was used for determination of the urease activity was as follows: The benzpyrene in caffeine and phosphate buffer solution was incubated together with catalase solution for 10 minutes, then urease solution was added, and the resulting clear solution was gently shaken for 1 hour to allow time for reaction between benzpyrene and the urease to occur.[2] Urea was then added, and, after a 15-minute incubation period, the reaction was stopped with 1 ml. of N sulfuric acid. All the incubations were conducted on a shaker at 37° C. A typical sample contained 1 ml. of 0.68 M phosphate buffer (pH 6.8), 1 ml. of 8 mg. per cent catalase solution, 1 ml. of urease, 1 ml. of 5 per cent urea, and 5 ml. of aqueous caffeine containing benzpyrene. Control incubations contained all the components above, except that benzpyrene was omitted from the aqueous caffeine solution. Ammonia produced in the sample by the action of urease was determined in two 1-ml. aliquots by direct Nesslerization. The intensity of color was measured on a Klett-Summerson colorimeter. Control incubations were conducted in triplicate, and the mean of the colorimeter values gave the total uninhibited activity of the urease. This value was used as a basis for calculating the urease inhibition in each benzpyrene-containing sample.

When *p*-chloromercuribenzoate was used in the incubated samples, it was added 40 minutes after the urease and 20 minutes before the urea. In every other respect the incubations were conducted in the manner previously described.

RESULTS

Preliminary studies of the effect of caffeine solutions of benzpyrene on Arlco jack bean urease[3] yielded results which were in accord with the findings of Mueller and Rusch (16). In the absence of catalase, it was found that the urease was inhibited to the extent of 15 per cent. In the presence of catalase, the urease activity was no longer inhibited by the benzpyrene, and, hence, the inhibition otherwise noted was probably due to the formation of small amounts of hydrogen peroxide.

In another preliminary experiment, Arlco urease powder was incubated with a solution of benzpyrene in caffeine. After a 22-hour incubation period in the dark, the ultraviolet absorption spectrum of the benzpyrene was unchanged. When the procedure of Miller (14) was used for isolating benzpyrene bound to protein, a very small amount of protein-bound material was detected by fluorometric analysis. This amounted to less than 0.005 per cent of the benzpyrene used.

TABLE 1

THE EFFECT OF BENZPYRENE ON UREASE*

Exp. no.	No. of samples	BP conc. in sample (mg/100 ml)	Per cent inhibition (av. [range])	BP sample used†
1	3	1.6	15(7–20)	B-1
	3	0.9	5(0–13)	
	3	0.6	2(− 2– 6)	
	3	0.3	2(− 1– 8)	
2	4	7.8	23(19–28)	B-1
	2	2.3	22	
	2	0.8	21	
	2	0.2	0	
3	4	0.9	32(26–39)	A
4	8	1.5	15(7–20)	B-1
5	10	1.5–1.9	− 2(− 8– 7)	B-2
6	6	1.0–1.2	12(8–15)	B-3

* The urease solution was a 1:350 dilution of the urease stock solution.
† A, B-1: Subject to ordinary laboratory lighting for several months.
B-2: B-1, after being kept in the dark 1 month.
B-3: B-2 after 2 weeks' exposure to fluorescent lights and sunlight.

When purified urease was used, the results differed markedly from those obtained with Arlco urease. Benzpyrene which had been kept in the solid state under ordinary laboratory lighting conditions (benzpyrene samples A, B-1, Table 1) regularly produced a 15–25 per cent inhibition of the urease activity in the presence of catalase. The results of Experiments 1 and 2 of Table 1 show the effect of the benzpyrene concentration on the urease inhibition. With the lower concentrations of benzpyrene, the inhibition was roughly proportional to the benzpyrene concentration. It appeared that a maximum inhibition was reached, however, beyond which further increases in the benzpyrene concentration would not increase the urease inhibition. Also shown in Table 1 are the results of a number of similar experiments, all of which show the urease-inhibiting activity of benzpyrene sample B-1. When three different solutions of this benzpyrene sample were kept in the dark for several weeks, their urease-inhibiting activity entirely disappeared.

Disappearance of urease-inhibiting activity was observed when solid benzpyrene was stored in the absence of light for a month (benzpyrene sample B-2). As seen in Table 1, Experiment 5, solutions

[2] A control experiment showed that when the preliminary incubation period was omitted, essentially the same results were obtained.

[3] Obtained from the Arlington Chemical Co.

prepared from this benzpyrene sample no longer produced any effect on the activity of the urease. However, after this inactive sample of benzpyrene had been exposed in a lime glass bottle to fluorescent lighting and to direct sunlight through window glass for a period of 2 weeks (benzpyrene sample B-3), it again inhibited urease activity.

The next series of experiments was designed to obtain further evidence for the implication of sulfhydryl groups of urease in the inhibitory effect of benzpyrene. Experiment 7 of Table 2 shows the

TABLE 2

THE EFFECT OF CYSTEINE ON THE INHIBITION OF UREASE BY BENZPYRENE*

Exp. no.	No. of samples	Ml. of 0.01 M cysteine	Per cent inhibition (av. [range])
7†	4	0	19(12– 26)
	3	1.0	1(0– 1)
8‡	3	0	20(15– 24)
	3	1.0	0(– 1–+ 1)

* The urease solutions used in Exp. 7 and Exp. 8 were 1:700 and 1:500 dilutions, respectively, of the urease stock solution.
Benzpyrene sample B-3 was used in these experiments.

† Cysteine added at the beginning of the incubation period.

‡ Cysteine added 40 minutes after the beginning of the incubation period.

effect of adding 1 ml. of 0.01 M cysteine to the sample at the beginning of the incubation period. It is apparent that there was no inhibition of the urease by the benzpyrene in the presence of cysteine.[4] When the benzpyrene and urease were incubated together for 40 minutes before the addition of the cysteine, no inhibition of the urease could be detected subsequently. Hence, cysteine not only prevents the inhibition of urease by benzpyrene but reverses the inhibition after it has occurred.

The addition of increasing amounts of *p*-chloromercuribenzoate to identical solutions of urease brings about a progressive inactivation of the urease molecule (10). This inactivation is due to a combination of the *p*-chloromercuribenzoate with the sulfhydryl groups of the urease. When this type of experiment was conducted with urease solutions in the presence or absence of benzpyrene, results were obtained which are shown in Table 3. The urease activity for each level of *p*-chloromercuribenzoate is definitely decreased by the presence of the benzpyrene.

DISCUSSION

The effect of light in altering the properties of benzpyrene solutions is well known. Irradiation of solutions of benzpyrene produces water-soluble materials which will kill infusorian coleps (15). Irradiated solutions of carcinogens will inhibit the growth of chick embryo cultures (12) and will cause the cytolysis of paramecia (4). Cysteine greatly increases the survival time of the paramecia. Irradiated solutions of carcinogens inhibit lactic and succinic dehydrogenases (3). When irradiation was carried out in oxygen-free solvents, there was no inhibition of these enzymes. This suggested that the photochemical reaction was of an oxidative nature.

The experiments presented in this paper emphasize in a quantitative manner the extreme reactivity of benzpyrene to light under the very mild conditions of low intensity exposure. The urease-inhibiting property of benzpyrene disappears when either the solid benzpyrene or solutions of benzpyrene are kept in the dark. There are at least two alternative explanations of this phenomenon. First, the photo-reaction product may be in equilibrium with benzpyrene and revert to it in the absence of light. An example of this type of reaction is the photochemical dimerization of anthracene (21). In the absence of light, the photochemical reaction product, dianthracene, dissociates to regenerate anthracene. On the other hand, the photochemical reaction product of the benzpyrene might be of a labile nature and decompose to an inactive material. Water-soluble photochem-

TABLE 3

THE EFFECT OF *p*-CHLOROMERCURIBENZOATE ON UREASE ACTIVITY IN THE PRESENCE OF 3,4-BENZPYRENE

Sample	No. of samples	Ml. of *p*-CMB*	Mean urease activity in Klett units
Control	3	0	212
Benzpyrene	3	0	169
Control	3	0.2	128
Benzpyrene	3	0.2	89
Control	3	0.35	90
Benzpyrene	3	0.35	53

* *p*-Chloromercuribenzoate.
The urease solution was a 1:500 dilution of the urease stock solution.

ical reaction products of carcinogens which are labile have been described (1). In the present study no attempt was made to remove oxygen from contact with benzpyrene in the solid state. Consequently, it is not known whether oxygen is involved in this instance in the production of the photochemical reaction product.

The results with cysteine and *p*-chloromercuribenzoic acid give definite evidence that the benzpyrene "covers" the sulfhydryl groups of the urease. Further evidence is needed, however, to

[4] As would be expected (10), the cysteine brought about an increase in the urease activity in both the control and the benzpyrene incubation samples.

establish the chemical nature of this "covering reaction."

SUMMARY

Benzpyrene which had been stored in the dark had no effect upon the activity of urease. The same benzpyrene which had been exposed in the solid state to ordinary laboratory illumination through a colorless glass bottle inhibited urease. This inhibitory activity disappeared when either the solid benzpyrene or solutions of benzpyrene were stored in the dark, and reappeared after the solid had been exposed to light again.

The activity of urease inhibitory benzpyrene was prevented by cysteine. This effect of cysteine, in conjunction with the results obtained after treatment of the urease samples with *p*-chloromercuribenzoate, indicates that the inhibitory effect of the benzpyrene was due to its action on the sulfhydryl groups of the urease.

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Insect Tumors Induced by Nerve Severance: Incidence and Mortality*

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Among the causative and contributory factors in abnormal growth, the nervous system deserves attention—even though, to date, very little experimental evidence is available to suggest a relationship between nervous disturbance and tumor development. The insect, *Leucophaea maderae*, is one example which illustrates such a relationship. In this species, tumors[1] can be induced experimentally by severance of the recurrent nerve. Branches of this autonomic nerve innervate the organs affected, i.e., the anterior portion of the alimentary canal (foregut and anterior midgut, or stomach) and the salivary organs (salivary glands and reservoir). This observation has been reported in preliminary form (1–3). A series of studies is intended to deal with certain aspects of this problem in somewhat more detail.

A large proportion of animals in which the recurrent nerve has been cut succumb to these tumors after varying periods of time. It had been noted early in this work that males and females show differences in their response to the development of tumors (4). The nature of these differences was analyzed in experiments to be reported here.

MATERIALS AND METHODS

Leucophaea, the insect used in this work, is a large, ovoviviparous roach belonging to the subfamily Panchlorinae. Of South American origin, the animal has been bred in the laboratory for over 10 years. Stock colonies are kept in large earthenware crocks; for experimental animals, pint-size glass jars are suitable. The food consists of apples, carrots, dog chow, and occasionally hard-boiled eggs. This insect offers many advantages as an experimental animal. Neither anesthesia nor sterile precautions are necessary for the operations performed. Postoperative losses are small. Since the normal life span of the species is long (2–3 years), there is ample time for tumors to develop.

In the experimental series on which this report is based, conditions were kept as constant as possible. The animals used for operations were "dated," i.e., the time of their emergence from nymphs into adults was recorded. All animals were young adults (not exceeding 48 days of "adult age") at the time of the nerve operation. As many suitable specimens as became available were subjected to nerve severance within approximately the same period of time. For reasons to be discussed later, the gonads were removed from about one-half of the group several weeks prior to nerve severance.

Thus, four groups of animals were available for comparison:

a) Females with nerve severance
b) Males with nerve severance
c) Castrate females with nerve severance
d) Castrate males with nerve severance

The animals were regularly checked. When a specimen had died or had to be sacrificed because it seemed close to death, it was autopsied, and the presence or absence of tumors was recorded. As "positive" were marked those animals in which at least one of the organs normally innervated by the recurrent nerve showed a tumor. There are no certain criteria by which the presence of tumors can be ascertained in living, unopened animals. In cases where the response to the nerve injury is slow, death may occur before tumor growth has proceeded to the point where it can be identified with certainty at autopsy. Even with daily checks for deaths in the tumor colonies, a small number of animals are unsuitable for histological examination, because the organs involved may show post mortem decay. Another small proportion of animals is lost in each experimental series for various reasons, such as escape, occasional cannibalism,[2] death from postoperative or unidentified causes.

RESULTS

Tumor Incidence

A precise determination of the incidence of insect tumors due to nerve severance meets with difficulties. For one, the interval between operation and death from the tumors varies widely—from a few days to several months. The longer the animals survive, the greater is their chance to be lost for reasons mentioned in the preceding chapter. Among the lost animals may be potential positive tumor cases. Since only those specimens in which

* Research grants from the American Cancer Society and The Anna Fuller Fund are gratefully acknowledged.

[1] The term "tumor" as used here does not imply that the growths under investigation are in every respect of the same nature as neoplasms in vertebrates. Histopathological and histophysiological data have been accumulated and will be reported in subsequent papers in which these growths will be characterized in detail and in which their differentiation from lesions such as injury reactions (6) will be discussed.

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[2] This difficulty could be avoided by placing experimental animals in individual containers. However, it has been found advantageous to keep two to three specimens together in order to promote activity, feeding, etc.

tumors could actually be identified are listed as positive in our experiments, the figures on tumor incidence obtained represent conservative values.

Of the 150 specimens in which the recurrent nerve had been cut, 95 were classified as positive, 39 negative, and 16 inconclusive—i.e., they were difficult to diagnose on account of post mortem changes, etc. The tumor incidence in this group was therefore 71 per cent (63 per cent for the males, 76 per cent for the females). Among the 189 gonadectomized specimens with nerve severance, 117 were positive, 39 negative, and 30 inconclusive. The tumor incidence for this group as a whole was 75 per cent (83 per cent for the males, 67 per cent for the females).

These values are in line with those of earlier experimental series in which the estimated incidence ranged from about 70 to 80 per cent. It can thus be stated that in the insect, *Leucophaea*, the incidence of tumors developing due to nerve severance, is high, i.e., roughly 75 per cent. Castration preceding nerve operation does not seem to influence the tumor incidence appreciably. Although it would appear that more castrate males develop tumors than castrate females and noncastrate males, these differences should not be considered significant unless they can be further substantiated. In part, they can be explained on the basis of differences in mortality rates, which will be discussed in the following section.

It has been mentioned that tumors develop in several locations below the level of nerve transection. Post mortem examination of a given specimen may show the presence of tumors either in one or more of the organs in question. The relative frequency with which each of these sites is involved differs markedly. These differences seem to depend, at least in part, on varying speeds of tumor growth, according to the organ affected, and on differences in the lethal properties of the various tumor types.

The site which was found most frequently affected was the midgut (stomach). Of 212 positive cases (with one or more tumor sites) in the present series of experiments, 160 had tumors in the midgut, 88 in the salivary organs, and ten in the foregut.

Mortality of Tumor-bearing Insects

It has been stated before that the time elapsing between nerve severance and death due to tumors varies considerably. Since the onset of tumor growth cannot be determined without sacrificing the animal, no precise statement can be made as to when tumors start to grow and how long a tumor-bearing animal can survive. It may well be that first signs of tumorous change appear soon after nerve severance in all specimens concerned. This process may lead to rapid tumor growth, in some cases causing early death, and may proceed so slowly in others that it does not become fatal before death from other causes intervenes.

For the duration of the survival period also the site of tumor formation is important. Tumors in the midgut tend to appear soon after operation and often develop so rapidly that death occurs before tumors in the salivary organs have a chance to develop. With increasing survival rates, therefore, the incidence of tumors in the salivary organs, particularly in the salivary reservoir, increases. Tumors in the salivary organs alone may or may not be lethal to the host. At any rate, in the experiments reported here midgut tumors must be considered the prime cause of death.

In an evaluation of the tumor mortality in *Leucophaea*, it is necessary, therefore, to focus attention on those tumor deaths that occur relatively soon after nerve severance. Consequently, it was decided to consider only those animals that died of tumors within 200 days after the nerve operation. Tumor-bearing animals that survived longer were not included in the analysis. Their tumors, being located for the most part in the salivary reservoirs, could not be considered responsible with reasonable certainty for death, in view of the survival rate of normal adults. To illustrate this point, in a group of 117 unoperated controls, the mean "adult age" (interval between emergence of the adult and death) was almost 13 months (387 days), with a maximum of 29 months in females and of 21 months in males. The gap between these normal survival rates and the "adult age" (a maximum of 48 days preceding nerve section plus up to 200 days postoperatively) of the tumor groups seems sufficiently large to minimize the danger of including in the analysis deaths not attributable to the tumors.

Among the animals in which the recurrent nerve had been severed, 54 tumor-bearing females and 28 tumor-bearing males died within the period of 200 days after the operation. The mortality rate in the two groups shows a marked difference in that the males survived considerably longer than the corresponding female group. For the male group, the mean survival time was 104.5 days, with a standard error of ± 11.43; that for the female group was 65.0 days, with a standard error of ± 6.71. Calculation of the standard error of the difference between these two means results in a critical ratio (C.R.) of 3, which indicates that such a situation would occur by chance alone 3 times in 1,000. The difference in survival time, therefore,

appears to be statistically significant.[3] The mortality rates are expressed on a percentual basis in Chart 1. The curves were obtained by plotting the percentage of survivors in each group at 10-day intervals.

These curves, as well as the mean values, clearly indicate a sex difference in tumor mortality. Special attention should be called to the first few weeks, which constitute the most significant period. Here the difference between the sexes is rather striking. While the first male tumor death did not occur until the 23d day after operation, ten females died of tumors between 2 and 23 days. The 2-day interval is remarkable in that it represents the earliest tumor death ever recorded in our work. The possibility that this case might have been a spontaneous tumor is exceedingly remote. Among approximately 500 normal adult animals examined, only two had "spontaneous" tumors, none of which had reached the size of those obtained experimentally by nerve severance.

The question arose whether or not the observed sex difference in tumor mortality had anything to do with hormonal differences. There is still no definite proof for the presence of sex hormones in insects, although there are certain indirect indications for it (5). The demonstration of a possible influence of the male and female gonads on resistance to tumors would in turn constitute additional indirect evidence for the existence of sex hormones in insects. Therefore, it seemed to be of interest to test the effect of castration on tumor mortality.

Gonadectomy was performed, in either newly emerged adults or old nymphs. In order to allow ample time for the effect of this operation to manifest itself, subsequent nerve severance was not performed until at least 3 weeks had passed after castration. As can be seen from Chart 1, the two curves representing the survival rates of tumor-bearing castrates are very similar and constitute values intermediate between those of noncastrate tumor-bearing males and females. The mean value for the survival rate of the male castrate group was 80.6 days (± 6.18), that for the female castrates, 86.0 days (± 7.40). The two values do not differ significantly (C.R. = 0.56; P = 0.58). For the difference between male castrate and male noncastrate tumor-bearing animals, the C.R. is 1.84 (P = 0.06), and for that between female castrate and female noncastrate tumor-bearing animals the C.R. is 2.10 (P = 0.04).

The comparison of the four experimental groups of animals studied leads to the following conclusion: Since tumor-bearing noncastrate males and females show a difference in survival rate which

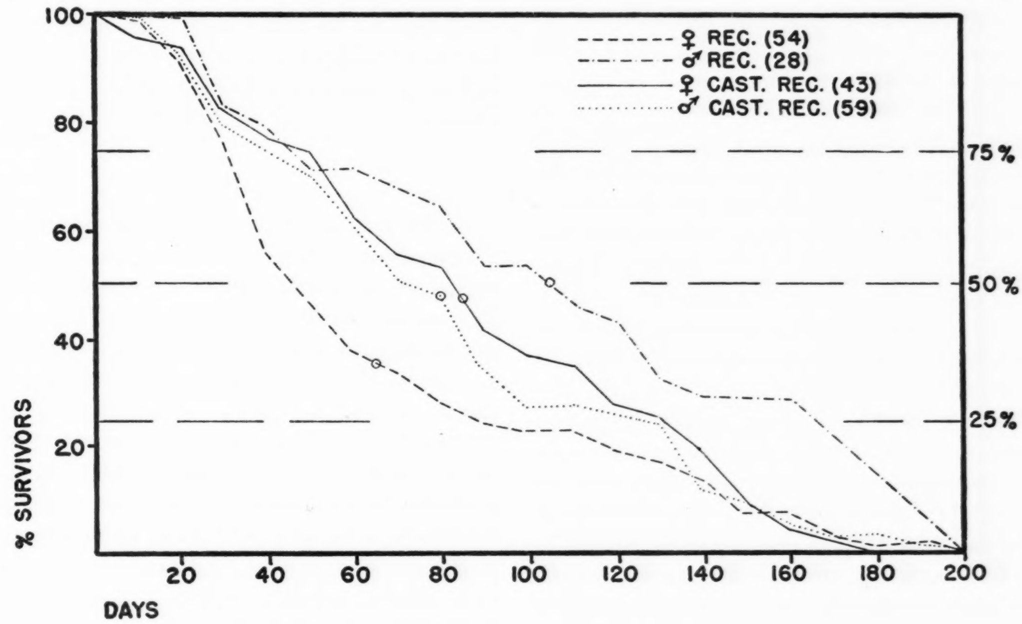

CHART 1.—Mortality rates of adult tumor-bearing Leucophaea males and females during a period of 200 days following nerve severance. The animals of the groups marked "REC." (54 females and 28 males) had the recurrent nerve cut on day 0. The animals of the groups marked "CAST. REC." (43 females and 59 males) had been castrated several weeks prior to day 0, on which nerve severance was performed. The curves show the survival rates of the four groups of animals on a percental basis. The means are indicated by small circles.

[3] I am grateful to Dr. Edith Boyd, Child Research Council, for her assistance in the statistical evaluation of these results.

can be abolished by castration, this difference must in some way be attributed to the gonads.

DISCUSSION

It has been shown that the presence of the sex glands in tumor-bearing insects seems responsible for differences in the mortality rates of males and females and that this difference disappears after gonadectomy. This result lends support to the assumption, so far only based on indirect evidence, that there are sex hormones in insects. A somewhat closer analysis of the survival rates in the four groups of experimental animals represented in Chart 1 shows that, while gonadectomy decreases the mortality in females, it increases it in males. The effect on the females might be explained in terms of metabolic changes. Normally, a substantial amount of reserve substances is needed for the development of the young. When the demand is abolished by ovariectomy, these substances become available for the needs of the female. These needs are increased by tumor development, which affects the vitally important alimentary canal. If tumor death were merely a consequence of starvation due to pathological changes in the gut, the change in tumor mortality after castration could be explained, at least as far as females are concerned, by changes in their metabolism, and no direct hormonal influences would need to be postulated. That the situation is not quite so simple is indicated, however, by metabolic studies which were undertaken to clarify this question (7). This work will be reported later in more detail. The results indicate that death in tumor-bearing females may occur at a time when their body reserves (especially fatty acids) are still intact. The causes of death must, therefore, be other than metabolic. In other words, in the female group the survival rate cannot be taken as a mere reflection of the body content in reserve material and cannot, therefore, be explained on this basis alone.

As far as the males are concerned, it is even more unlikely that metabolic changes caused by castration should themselves be responsible for the decrease in resistance to tumor growth. Even if changes in metabolism could be made responsible for the changed tumor resistance due to castration, the manner in which these metabolic changes are brought about suggests for its explanation the action of sex hormones.

Little need be said about the reported data on tumor incidence. They indicate that a large proportion, approximately $\frac{3}{4}$, of the specimens in which the recurrent nerve has been severed succumb to tumors. Evidence is not sufficient to postulate that the sexes differ with respect to the number of induced tumor cases. The seemingly higher incidence in females as compared to males of the same noncastrate group could be explained, at least in part, on the basis of the shorter survival time in females, which permits the identification of tumor cases with a somewhat greater degree of certainty. Furthermore, the observation that castration has no essential influence on tumor incidence makes a sex-linked difference in tumor susceptibility unlikely.

The higher frequency of tumors in the midgut, as compared to other locations, may be due to the fact that midgut tumors develop faster and reach lethal proportions before other tumors had a chance to manifest themselves.

SUMMARY

1. In the insect *Leucophaea maderae* the incidence of tumors developing after severance of the recurrent nerve is high—i.e., roughly 75 per cent. The incidence does not seem to differ significantly in males and females. Accordingly, castrates respond to the nerve transection in approximately the same way as noncastrate animals. Among the organs involved tumors appear most frequently in the midgut (stomach), an observation which may perhaps be explained by a more rapid and more destructive growth pattern of these as compared to tumors in other sites (salivary organs, foregut).

2. The mortality rates of tumor-bearing animals differ significantly: males as a group survive longer than females. The sex difference in resistance to the presence of tumors disappears after castration. This observation offers indirect evidence, but no proof, for the presence of sex hormones in insects.

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The Inhibition of Liver Carcinogenesis with 3′-Methyl-4-dimethylaminoazobenzene in Hypophysectomized Rats*

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From studies carried out at the University of California, a diversity of neoplasms has been observed in female rats given pituitary growth hormone for a prolonged period (6, 9–11). An absence of tumors, however, was noted in hypophysectomized rats similarly treated with the growth hormone (12). These investigators have also reported that the administration of methylcholanthrene to hypophysectomized rats resulted in fewer tumors than were obtained in control intact animals (13).

Many factors are known that alter the carcinogenic activity of the azo dyes; however, these studies have not been extended to hypophysectomized rats. Riboflavin and other dietary constituents will modify the action of certain of these compounds. Richardson and Cunningham (14) demonstrated that application of methylcholanthrene resulted in a lowering of the liver tumor incidence in rats fed diets containing 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB). Symeonidis *et al.* (16) reported that adrenalectomized rats fed diets containing 4-dimethylaminoazobenzene had a lower liver tumor incidence than did the normal controls. In addition, these workers observed that subcutaneous administration of desoxycorticosterone acetate resulted in a protection against liver damage by the azo dye in both intact and adrenalectomized rats. Recently, it was found in this laboratory that administration of the nitrogen mustard, methylbis(β-chloroethyl)amine, also resulted in a considerably lower liver tumor incidence in rats fed diets containing 3′-Me-DAB (3).

The present study was initiated to determine whether hypophysectomy would alter the activity of the active azo compound, 3′-Me-DAB, and to ascertain whether the above effects are related to pituitary or possibly pituitary-adrenal function.

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METHODS

The study was carried out in two parts. For the first series, male albino rats of the Holtzman-Sprague Dawley strain were hypophysectomized in this laboratory. Following this operation, the animals were maintained on Purina Laboratory Chow for 1 week. Rats, also males, of the second series were hypophysectomized by the Hormone Assay Laboratories, Inc., Chicago. Approximately 2 weeks intervened between the time of hypophysectomy and the start of the dye feeding in this series. All the animals were kept in wire-bottomed cages in a small incubated room maintained between 24° and 27° C. The synthetic diet used in this laboratory (4) contained 0.06 per cent 3′-Me-DAB and was fed ad libitum. Food intakes and body weights were taken at weekly intervals.

A few rats from each series died in the period between hypophysectomy and the initiation of feeding of the diet containing the azo compound. The survival during the actual study was remarkably good. Twenty-six animals were started on the diet containing the dye, and twenty-four survived throughout the study. At the time of sacrifice the animals were anesthetized with ether, the pleural cavity exposed, and the heart punctured. The adrenals, kidneys, hypothalamus, tongue, glottis, upper trachea, lungs, section of small intestine and stomach, testis, pituitary (if any remained), and a portion of liver or liver tumors, if present, were preserved in 10 per cent formalin. Dr. H. L. Richardson kindly agreed to carry out the histology and pathology on the two experimental series. The remaining liver tissues were homogenized in cold distilled water and were subsequently analyzed for riboflavin and desoxyribonucleic acid by methods routinely used in this laboratory (4).

RESULTS AND DISCUSSION

Results obtained in this study indicate that pituitary or possibly adrenal-pituitary function is involved in the induction of liver tumors by the carcinogenic azo compounds. Hypophysectomy effectively inhibited the formation of hepatomas in rats fed diets containing 0.06 per cent 3′-Me-DAB up to periods of 19 weeks; this period is almost twice that required to induce a 100 per cent tumor incidence in intact animals. The findings obtained thus far do not conclusively demonstrate that the hypophysectomized rats were given complete protection against the carcinogen. The feeding of the azo dye for prolonged periods would be required before it could be ascertained whether complete protection was provided or whether the process

was merely slowed down. From Table 1 it may be observed that the correlation between hypophysectomy and the protection against azo dye liver damage was excellent. Of the eighteen rats completely hypophysectomized which were sacrificed between 14 and 19 weeks only one (Rat R) showed any evidence of cirrhosis. All the others had normal appearing livers. In addition, the concentration of riboflavin and desoxyribonucleic acid in the livers of these animals was well within the normal range. The concentration of desoxyribonucleic acid in the livers of these animals fell between 320 and 380 mg/100 gm while the riboflavin values were between 18 and 20 μg/gm of liver.

the livers were severely cirrhotic and enlarged. Attention should be called to three of the animals in this series, F, I, and Q (Table 1), which exhibited mild cirrhosis at the time of sacrifice. In all these animals there was evidence of incomplete hypophysectomy. Whether or not the remaining pituitary was functional, which would enhance the action of the azo dye, could only be ascertained by a far more extensive study. All the results obtained, however, agree with the assumption that pituitary or adrenal-pituitary function is involved in the induction of liver tumors by the azo compounds. Livers from the partially hypophysectomized animals had a high concentration of desoxyribonucleic acid and a lowered concentration of riboflavin, which is characteristic when the azo dye is fed to intact rats for several weeks (4).

TABLE 1

THE EFFECT OF HYPOPHYSECTOMY ON THE CARCINOGENIC ACTION OF 3-METHYL-4-DIMETHYLAMINOAZOBENZENE

RAT NO.	TIME DYE WAS FED (Weeks)	APPEARANCE OF LIVER AT END OF DYE FEEDING	REMARKS
		First series*	
A, C Controls	14	Severe cirrhosis, large multiple tumors in each liver	Hypophysis intact, adrenals and gonads normal
B, D	14	Normal	Hypophysectomy complete, adrenal and testicular atrophy
F, I	16	Mild cirrhosis	Approximately 25 per cent of hypophysis left intact
G, H, J, K, L	16	Normal	All completely hypophysectomized, adrenal and testicular atrophy
		Second series†	
E	9	Normal	Complete hypophysectomy
M, N, O, P, S	15	Normal	Complete hypophysectomy
Q	19	Mild cirrhosis, no tumor	Pituitary tag intact, adrenals not completely atrophied
R	19	Mild cirrhosis, no tumor	Complete hypophysectomy
T, U, V, W, X	19	Normal	Complete hypophysectomy

* Holtzman-Sprague Dawley strain, hypophysectomized at Stanford. Average rat weight at start of experiment, 180 gm.; final weight, 165 gm. Average food intake, 9 gm/rat/day.

† Sprague-Dawley strain. Hypophysectomized at Hormone Assay Laboratories, Inc., Chicago. Average weight at start of experiment, 155 gm., final weight, 143 gm. Average food intake, 7.2 gm/rat/day.

In contrast to the above findings, investigators at the University of Wisconsin have conclusively demonstrated that the feeding of diets containing at least 0.048 per cent of 3′-Me-DAB to intact rats for 10–12 weeks resulted in a high percentage of hepatomas (1, 8). When diets containing 0.032 per cent or 0.04 per cent of this carcinogen were fed for 4 months they also induced a high tumor incidence (2). Cirrhosis and liver damage were evident in most of the animals fed the above diets. In this laboratory we have repeatedly observed that the feeding of diets containing 0.06 per cent 3′-Me-DAB for 12 weeks resulted in nearly a 100 per cent tumor incidence. Since the carcinogenic activity of this compound has been firmly established, only two rats were maintained for controls (A and C, Table 1). These animals were subjected to the surgical procedures of hypophysectomy, except that the pituitary was left intact. Under identical conditions these animals developed large tumors, and

It is difficult to provide a mechanism which would accurately explain the inhibitory action of hypophysectomy on the induction by the azo dye of liver tumors. Considerable concern has been given to the food intake of the hypophysectomized animals in this study. The food intake was lower in these animals than has been observed in intact rats. Animals of the first series had an average food intake over the dye feeding period of 9 gm/rat/day. In the second series, the value was 7.2 gm. From our past observations, 10 gm. daily per intact rat would constitute an average intake under comparable conditions to those in the present study. We are not of the opinion that the somewhat lower intake in the hypophysectomized rats could account for the almost complete absence of liver damage after almost 19 weeks of feeding the

diets containing 0.06 per cent 3′-Me-DAB. At the termination of the experiment, the weights of the hypophysectomized rats averaged within 15 gm. of their initial value; this finding also suggests that they were not in a state of inanition. The multiple functions of the hypophysis complicate any explanation of the results obtained. From the studies of Moon and associates (6, 9–11) it is suggestive that somatrophin is involved in cancer induction. On the other hand the induction of tumors by this hormone was effectively blocked by hypophysectomy. The thyroid hormone, gonadotrophins, or the lactogenic hormone also may be involved in the action of azo dye compounds. Carbohydrate metabolism likewise is influenced by the hormones of the adrenal and pituitary, which could alter the carcinogenic activity of the azo dyes. It is not possible to make reference to or to attempt any correlation with the many publications on hormones and cancer induction. Symeonidis and associates (16) have found that adrenalectomy or the administration of DOCA resulted in a lowering of the incidence of liver tumors in rats fed 4-dimethylaminoazobenzene. Kaplan (5) has conclusively demonstrated that adrenalectomy caused an increase in lymphoid tumors in C57BL mice exposed to total-body irradiation. Administration of cortisone concurrently with or after total-body irradiation inhibited the development of lymphoid tumors in this strain of mice. In studies on the inhibition of azo dye liver tumor formation by methylcholanthrene, Richardson noted that there was considerable adrenal change when the hydrocarbon and 3′-Me-DAB were administered together (15). This investigator[1] is of the opinion that the mechanism of inhibition of azo dye carcinogensis by methylcholanthrene must be through a block of the adrenal steroid mechanism between the hypophysis and adrenal gland. A similar explanation may possibly account for the inhibitory action of nitrogen mustard on 3′-Me-DAB carcinogenesis observed in this laboratory (3). Ludewig and Chanutin (7) have found that administration of the nitrogen mustards to rats caused adrenal hypertrophy and a loss of esterified cholesterol from the gland.

From the results of the present investigation we may conclude that the pituitary or possibly the adrenal, or both, is involved in azo dye liver carcinogenesis. Gonadotrophin, adrenocorticotrophin, cortisone, and other hormones will be administered to hypophysectomized rats fed diets containing this carcinogenic azo dye to determine, if possible, the function of the pituitary that is involved in this inhibition of carcinogenesis.

[1] Dr. H. L. Richardson, private communication.

SUMMARY

Hypophysectomy effectively inhibited the formation of liver damage and hepatomas in rats fed diets containing 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene up to periods of 19 weeks. In other experiments a 10–12-week period of feeding has usually been sufficient to induce a high percentage of tumors in intact animals.

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Regeneration of Mouse Liver after Partial Hepatectomy*

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The rapidly growing liver tissue after partial hepatectomy has been used advantageously by investigators to study changes during accelerated growth and proliferation of cells (4, 7, 12, 15). Previous reports have been chiefly on rats. In many instances, pooled samples were taken for chemical data. In the present investigation a comprehensive study was made on individual samples of regenerating (restoring) mouse liver by several methods of approach: (*a*) quantitative morphological and cytological studies of cell and tissue growth (22), (*b*) histochemical and cytochemical localization of substances (23), and (*c*) quantitative biochemical analyses (20).

MATERIALS AND METHODS

A total of 165 white mice of strain A was used in the preliminary and final experiments. Three-month-old male animals weighing 24–27.5 gm. were fed ad libitum, because fasting, a procedure used on rats by some investigators, tended to increase the mortality rate in mice.

Partial hepatectomy was performed by a single person to reduce possible variations in technic. The method of Brues, Drury, and Brues (4) was followed with slight modifications. The left lateral and median lobes were ligated at the base and excised under ether anesthesia, with special attention being given to tying the ligature at the proper level to avoid obstruction of the hepatic portal vein and the bile duct. Hemostats were not used. The percentage of total liver tissue removed was determined in a preliminary experiment by operating on 29 male mice by the standard procedure and weighing both the excised and remaining portions immediately. Results showed that 65 per cent (standard deviation ± 4) of the total liver was removed by partial hepatectomy (Table 1). Thus, the weight of the excised portion gives an indication of total liver weight. In the final experiments only those animals with an excised liver weight of more than 0.84 gm. were used, with a range in weight from 0.84 to 1.00 gm. in 90 per cent of the animals. This was done to select a reasonably uniform group of experimental animals.

After partial hepatectomy, changes in body weight were followed as an indication of postoperative health. Regenerating livers were selected from animals showing the least body weight loss soon after hepatectomy and a steady gain in weight in later stages. The residual lobes were always removed between 9:00 and 10:30 A.M., and all analyses made on individual animals. For microscopic studies pieces of tissue were collected immediately from the posterior lobule of the right lateral lobe for freezing-drying (16) and chemical fixation. The remainder of the right lateral lobe and caudate lobe was used for biochemical analyses. Desoxyribonucleic acid, nitrogen, and other chemical constituents were determined by the methods previously described (19).

Observations were made on the regenerating liver at the following intervals after partial hepatectomy: ¾, 1, 2, 3, 4, 5, 6, 7, 8, 10, 14, 21, 28, 38–45 days, and 2, 4, and 6 months. Six to nine animals were used in each group, except after 45 days when four to five animals were examined. Two types of control material were used: (*a*) livers from normal healthy males of 3 months (ten animals), 5 months (four animals), and 9 months (five animals) for comparison with early and late stages of restoration after partial hepatectomy; and (*b*) for some animals, the excised portions were analyzed to serve as their own controls.

A brief study was made comparing the two methods available for obtaining nuclear counts: (*a*) that of counting the nuclei from microscopic sections (4); and (*b*) using a diluted sample of the fresh homogenate in a counting chamber (12). The former method proved to be extremely tedious and time-consuming, with more possible sources of error than the latter. The counts from sections were usually slightly higher than those obtained from fresh homogenates. The latter method was adopted for our experiments. A homogenate was prepared for each liver in an all-glass homogenizer (Scientific Glass Apparatus Co.) with 9 ml. of slightly alkaline 0.85 per cent sodium chloride solution per gram of tissue for quantitative chemical analyses. For counting purposes, 1 ml. of this sample was diluted further with 19 ml. of saline. This was mixed with an equal part of a staining solution, which consisted of 80 mg. crystal violet in 100 ml. of 6 per cent acetic acid. Nuclei can be distinguished readily with this staining mixture.

Samples were agitated on a variable speed shaker (Eberbach & Sons) and counted within 2 hours after preparation. Repeated counts made at intervals within this period showed no change. In comparing different dyes at various concentrations it was observed that homogenates prepared with saline gave distinctly darker staining reactions than those with 0.88 M sucrose.

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Counts were made with the hemocytometer, using five 1-mm. squares from two chambers. The original counts in this volume of fluid (1 c. mm. in the ten squares) ranged from 275 to 600 nuclei, the lower figures for some of the regenerating livers and the higher numbers for control livers. Each sample was counted by two persons whose average was accepted when their individual results were within 5 per cent of each other's counts. When not in agreement, the counts were repeated until agreement was obtained. Nuclei belonging to all types of cells (parenchymal, blood, smooth muscle, connective tissue, bile duct, Kupffer cells, and macrophages) were included, since they contribute to the quantitative values of desoxyribonucleic acid.

TABLE 1

QUANTITATIVE RELATIONSHIP BETWEEN LIVER AND BODY WEIGHTS AND NUMBER OF NUCLEI

	Liver weight (gm.)	Percentage liver/body weight	No. nuclei per gm.$\times 10^{-6}$
Regenerating liver:			
$\frac{3}{4}$ day	0.67	2.7 (2.5–2.8)	146 (110–163)
1 "	0.70	3.0 (2.7–3.6)	156 (149–165)
2 days	0.81	3.5 (2.8–4.1)	128 (116–138)
3 "	0.89	3.9 (2.8–4.6)	137 (109–154)
4 "	1.08	4.7 (3.6–5.5)	133 (112–163)
5 "	1.12	4.9 (3.8–5.9)	144 (87–294)
6 "	1.23	5.4 (5.0–5.8)	147 (107–165)
7 "	1.20	5.1 (3.5–5.9)	156 (134–189)
8 "	1.43	6.4 (5.3–7.5)	126 (116–134)
10 "	1.38	5.8 (4.4–7.4)	148 (132–167)
14 "	1.56	6.3 (4.6–7.6)	133 (112–146)
21 "	1.46	5.7 (4.7–6.8)	147 (123–162)
28 "	1.55	6.0 (4.8–9.0)	176 (150–198)
38–45 "	1.54	5.5 (4.5–7.8)	172 (141–195)
2 months	1.38	5.2 (4.7–6.4)	166 (142–216)
4 "	1.28	4.3 (4.1–4.6)	219 (186–258)
6 "	1.75	5.6 (4.8–6.6)	171 (155–195)
Controls:			
Before hepatectomy	1.43	5.5 (4.1–6.2)	
After hepatectomy	0.50	2.0 (1.4–2.5)	
Normal controls 3-mo. males	1.45	5.4 (4.9–5.9)	215 (204–231)
Normal controls 5-mo. males	1.49	5.5 (4.5–7.3)	174 (159–192)
Normal controls 9-mo. males	1.40	5.1 (4.5–5.8)	213 (192–237)

Tissues fixed in Zenker-acetic fluid were cut at 4 μ after paraffin imbedding. These were stained with hematoxylin-eosin for histological observations. Material from control animals as well as the excised control and regenerated portions was examined.

The percentage of parenchymal cells in mitotic division was determined as described in the following paper (22). The percentages of binucleate parenchymal cells and other cellular elements in the liver tissue were obtained at the same time the mitotic counts were made. Results from the 4-μ sections compared favorably with those taken from 12-μ sections, except for slight discrepancies in instances where the liver cells were much enlarged, as in some regenerating livers. Here, as expected, the relative number of parenchymal nuclei tends to be higher in the thinner section. Since it was not feasible to make separate counts from 12-μ sections for the entire experiment, all counts to be reported were obtained from 4-μ sections. No correction factor for section thickness was introduced in calculations of percentages.

OBSERVATIONS

Partial extirpation of the mouse liver results in a rapid growth of the remaining lobes in a manner similar to those of other rodents. Although an effort was made to control many variable factors, mice do not respond to partial hepatectomy in an entirely uniform manner with respect to any of the criteria observed. Although some attempt will be made to indicate the degree of individual variation in some of the data, generally the mean group trends will be discussed. Changes in body weight, liver weight and cell population, and some of the chemical results will be presented here. Further information on biochemical, histochemical, and quantitative cytological studies will appear in subsequent papers (20, 22, 23).

Liver and body weight changes.—Following partial hepatectomy there is a loss in body weight exceeding the immediate loss of 3.6 per cent due to removal of the two lobes of liver. On the first day the average body weight drops to 8 per cent below that immediately after hepatectomy, with little change through the tenth day. Although a few animals begin gaining weight on the fourth day, it is not until the fourteenth day that the average weight shows an increase. A steady gain in weight is observed thereafter, and complete recovery to the pre-operative range occurs at 21 days.

The right lateral and caudate lobes show a rapid increase in weight soon after hepatectomy, so that the original total liver weight is regained by the eighth day (Table 1). This quick growth, coupled with a loss in body weight during this period of early regeneration, tends to bring the percentage of liver to body weight back within the normal range much sooner than if the body weight were to remain normal. The largest gain of 26 per cent in this ratio occurs during the first 18 hours; by the end of the first day there is an increase of 33 per cent over that of the hepatectomized controls. The normal pre-operative ratio is restored as early as the fourth day in some animals, but the average ratio is not back to normal until the sixth day. Since the increase in weight of the liver remnants continues while the restoration of the body weight lags, the ratio is higher than normal between the eighth and 28th days.

Much of the early gain in liver weight is due to mobilization of lipids into parenchymal cells and increase of fluid in the liver as evidenced by histochemical and chemical observations. The total nitrogen and desoxyribonucleic acid content increases only slightly during the first day when synthesis is presumably not occurring at a fast rate. Restoration of these constituents progresses rapidly from the second to the eighth day, their

curves paralleling that of liver weight (Chart 1). The second day must necessarily be the period of most active premitotic synthesis of substances concerned in the duplication of chromosomes and production of daughter cells as will be discussed below.

Changes in number of nuclei.—The number of cells begins to increase slowly from the second day when some individuals first show increased mitotic activity. A peak in mitotic activity of parenchymal cells is reached on the third day, at which time an average of 6.7 per cent of these cells is found to be in some stage of mitosis (22). A noticeable increase in the number of cells is therefore expected to occur on the fourth day. Actually, the total number of nuclei increases from 121 to 145 million (17 per cent) between the third and fourth days. This is

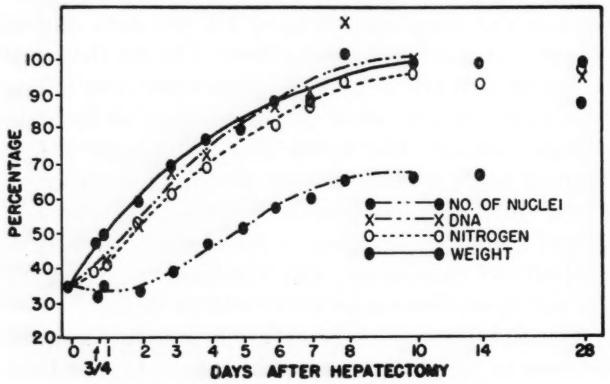

CHART 1.—Average percentages, as compared to normal controls, of total numbers of nuclei, desoxypentosenucleic acid, total nitrogen, and weight of liver tissue within groups of mice at intervals after partial hepatectomy.

the largest daily percental increase during the period of early regeneration. The marked increase in liver to body weight ratio at this time thus represents an actual increase in number and growth of cells. After the third day mitotic division declines gradually, and by the seventh day rapid proliferation is essentially over. Following this, the number of cells apparently continues to increase slowly by some mitotic activity, which, however, is so low that only a few are observed in the size samples used. The total number of nuclei reaches 87 per cent of the normal value at 28 days after hepatectomy (Chart 1). At 2, 4, and 6 months, however, the numbers are 88, 92, and 101 per cent, respectively, when compared to control animals of 5 and 9 months of age. The number of nuclei per gram of tissue is low through the 21st day (Table 1), indicating that the average size of cells in the regenerating liver is somewhat larger than in the controls.

Mitotic activity of the lining cells and bile duct cells occurs particularly during the first several days of regeneration, although counts were not made for these. Increase of bile duct cells is accompanied by multiplication of the underlying connective tissue cells.

The percentage of parenchymal cell nuclei in the liver tissue was obtained from sections at the same time that counts were made for mitotic cells. In the normal mouse liver an average of only 56 per cent of the total nuclei belong to parenchymal cells, the remainder belonging to bile duct, Kupffer, connective tissue, smooth muscle, and mesothelial cells and also to macrophages and white blood cells. The percentage of nuclei other than those of hepatic parenchymal cells at various stages of regeneration are presented in Charts 2 and 3. The range for each group has also been indicated. There is an increase of the nonparenchymal elements from the third through the 45th day of liver restoration. This increase may be due to one of three conditions or a combination of all these factors: (*a*) a more rapid proliferation of cells other than parenchymal cells, (*b*) a decrease in the number of parenchymal cells due to some focal necrosis, and (*c*) an infiltration of leukocytes and macrophages accompanying focal necrosis.

The numbers of liver parenchymal nuclei were calculated from total nuclear counts, using the percentages of parenchymal nuclei obtained from microscopic sections. This revealed that at 7, 14, 21, and 28 days of regeneration the liver parenchymal nuclei recovered are 48, 54, 58, and 66 per cent, respectively, of the normal controls. At 6 months the number returns to 99 per cent of the original. Correction of these numbers for binucleate cells gives the total number of liver parenchymal cells recovered at various intervals. At 7, 14, 21, and 28 days the numbers of liver cells are, respectively, 58, 63, 68, and 76 per cent of the control livers. This rises to 95 per cent at 4 months and 114 per cent at 6 months. These represent relative gains only, since the percentages are derived from calculations of several sets of data.

Histological findings.—Individual variations are found in control livers as to the amounts of leukocytic infiltration, glycogen content, and necrotic cells. Of the ten normal animals of 3 months, three showed slight leukocytic infiltration around the portal or central veins. Since the animals were not fasted, there were varying degrees of glycogen storage, the areas occupied by glycogen in the living cells appearing as unstained areas in hematoxylin-eosin slides. These observations on glycogen were substantiated by histochemical tests and chemical determinations. One control sample contained a few enlarged nucleoli, which have been designated by some authors as nuclear inclusion

bodies. The physiological significance of these abnormally enlarged nucleoli has not yet been elucidated.

The excised control portion for each animal was examined closely for indications that might explain the wide individual variations in response after partial hepatectomy. In addition to the differences observed above for control animals, this survey of excised livers revealed that liver tissue from 14 per cent of the animals contained small areas of focal necrosis or isolated necrotic cells. This observation, however, does not necessarily mean that these particular animals were predisposed to develop necrosis in the regenerating portions. During the first 10 days, focal necrosis was evident in only five regenerating livers from eleven animals which originally showed necrotic cells, either singly or in small groups. Larger numbers of regenerating livers showing focal necrosis were from animals whose excised portions were normal in appearance.

At 18 hours after partial hepatectomy, some livers showed evidence of engorgement and dilatation of the sinusoids and blood vessels as well as hemorrhage and thrombi in sinusoids. Some focal necrosis was observed principally in the mid-zonal areas. The occurrence and extent of these necrotic areas varied considerably among individuals. This change was followed by some infiltration of leukocytes. The parenchymal cytoplasm appeared vacuolated during the first few days, due to large amounts of lipids. These vacuoles stained positively for lipids with oil red O in formalin-fixed material.

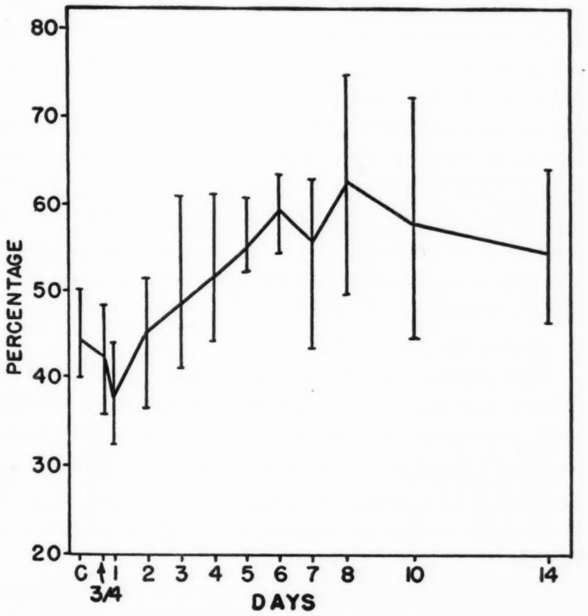

CHART 2.—Percentages of mouse liver nuclei that are not in hepatic parenchymal cells at intervals during regeneration following partial hepatectomy.

The occurrence of focal necrosis at $\frac{3}{4}$ and 1 day appeared to be due to some difficulty in the vascular channels, since there was evidence of engorgement in larger blood vessels. Following this there was a period of repair, so that at 2 days all nine animals showed either areas of resorption of necrosis or no involvement. At 3 days, however, one-third of all the regenerating livers again contained some groups of necrotic cells. The period in which the largest number of animals showed recent hemorrhage, necrosis, and fibrosis was between the fifth and tenth days. Some necrosis was still evident at 28 days in two out of six individuals. However, at 2, 4, and 6 months this process had ceased completely.

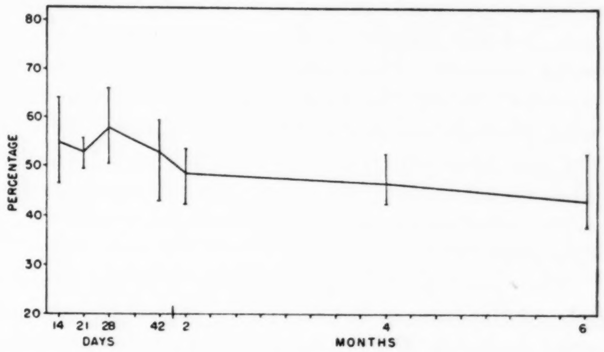

CHART 3.—Percentages of mouse liver nuclei that are not in hepatic parenchymal cells at intervals during regeneration following partial hepatectomy.

DISCUSSION

The restoration of mouse liver proceeds at a variable rate in different individuals. Apparently, the time required for readjustment of the remaining tissue prior to cell proliferation and actual growth depends on physiological factors which are difficult to control in these experimental animals. Animals in this experiment were selected as to sex, age, body weight, weight of excised liver, and also on the basis of good postoperative health. Tissues were collected within the same time period in the morning. These factors did not eliminate the wide range in variations of results. Animals which responded quickly showed mitotic activity at 48 hours or earlier, while some showed no mitosis at this time. Fasting the animals prior to operation would probably not have diminished the variations, since Brues and Marble (5), using fasted rats, obtained similar variations in mitotic activity. It is clear from the present study that the length of time elapsed after partial hepatectomy is not necessarily indicative of the degree of restoration occurring in individual livers. Unfortunately

a better criterion for different stages of restoration could not be used, although attempts were made at grouping animals according to the percentage of mitotic activity, amounts of desoxypentosenucleic acid, protein nitrogen, and other criteria. Using mitotic activity as an index of the degree of regeneration creates problems in grouping animals after the initial stages of proliferation, since mitotic activity continues variably for several days.

The initial adjustment to the great loss of functional tissue is that of mobilization of lipids and other constituents into the liver with concomitant loss in body weight, indicating a heavy drain on body reserves. The remarkable increase in lipid content during the first 2 days is in agreement with histochemical and chemical observations on the rat liver after partial hepatectomy (15, 18). This increase in lipids is followed by an influx of other substances in preparation for synthesis of new cells (20).

The removal of the gall bladder with the median lobe at the time of hepatectomy probably causes a greater degree of disturbance in the biliary system of the mouse than in the rat, which lacks a gall bladder. Whether this causes an actual delay in regeneration is not known.

In this experiment the young adult mouse liver showed a peak in mitotic activity on the third day of regeneration. In rats this has been reported to occur at 24 hours (5), at 30 hours (15), and on the second and third days (12). A direct comparison cannot be made between these different groups of animals, since, among other factors, the age of the animal affects the rate of regeneration (6, 8, 9). There is an earlier and greater restoration of mass and number of cells in young animals compared to adult and old individuals. There may also be differences in response between different strains of the same species.

The early changes in the first few days are of chief interest to investigators concerned with processes accompanying rapid proliferation of cells. The premitotic influx of various substances, the period of synthesis (cell division), and the postmitotic reorganization and synthesis of material for normal function occur within a relatively short time. A study of this sort, however, is at best one that shows an average activity of large numbers of cells which are in different stages at any one time. At the height of mitotic activity, for example, an average of only 6.7 per cent of the parenchymal cells was in division, the remainder of cells being either in premitotic or postmitotic periods or in a normal functional state.

In terms of liver to body weight ratio, the mouse liver is essentially restored to the original mass by the sixth day. The original total liver weight, nitrogen, and desoxyribonucleic acid, however, do not reach the control levels until the eighth day. Presumably, most of the functional cytoplasm is restored between the seventh and tenth days. The total number of nuclei, on the other hand, is slow to recover. There may be several factors which affect the number of nuclei obtained. First, it has been observed in this study and others (6, 13, 17, 22) that the number of binucleate cells is reduced during regeneration. This indicates mitotic activity of binucleate cells which form single metaphase plates with production of mononucleate polyploid daughter cells (3). Such cell divisions would then produce no net increase in the number of nuclei. There is ample evidence of increase in polyploid nuclei in regenerating livers (17, 21, 22). Second, some nuclei of the regenerating livers may have been fragmented in the process of homogenization and thus not included in the counts. Third, the functional tissue may have been restored by means of larger cells, without necessarily regaining the original number of cells.

The normal young adult mouse liver contains a large percentage of nonparenchymal cells. On the basis of nuclear counts on sections, only 56 per cent of all the nuclei in the liver tissue belong to parenchymal cells, although the volume occupied by these cells is 88 per cent (22). Siess and Stegmann (14) reported somewhat similar ratios of parenchymal to endothelial cells. Swift (11) reported that in beef liver the percentage of parenchymal nuclei is higher (70 per cent). Unpublished observations in this laboratory show similar results for the rat liver.

The relative as well as absolute number of parenchymal nuclei is reduced during regeneration, with a reciprocal rise in the number of nuclei belonging to nonparenchymal cells. Abercrombie and Harkness (1) observed a significant increase of littoral cells on the seventh day of regeneration in the rat liver.

The occurrence of necrotic cells in normal mouse liver has previously been reported by Wilson and Leduc (21) and Olitsky and Casals (10). Abnormal appearance of liver cell cytoplasm in early regenerating liver has been observed by Price and Laird (12), who described cytoplasmic inclusion bodies in the rat liver which appeared as early as 6 hours after partial hepatectomy and tended to disappear at 48 hours. Aterman (2) reported the presence of glycogen-free, fat-free vacuoles in rat liver as early as 5 minutes after partial hepatectomy. He believed that this "watery vacuolation" after hepatectomy is identical with that observed in the liver in anoxic states.

SUMMARY

Extirpation of 65 per cent of the mouse liver results in rapid growth of the remaining lobes, so that the original mass, in terms of percentage of liver to body weight, is essentially restored by the sixth day. However, the original total liver weight, nitrogen, and desoxyribonucleic acid are not completely recovered until the eighth day. The total number of nuclei increases more slowly and reaches 87 per cent of the normal value at 28 days after hepatectomy.

Parenchymal nuclei comprise only 56 per cent of the total nuclei of normal mouse liver, the remainder belonging to bile duct, Kupffer, connective tissue, smooth muscle, and mesothelial cells and also to macrophages and leukocytes. These nonparenchymal elements are increased between the third and 45th days of regeneration.

Substantial individual variation is observed in the response of mice to partial hepatectomy. The time interval after operation is not a satisfactory, uniform criterion of the degree of restoration of the liver remnant in individual mice.

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Cytological Changes in Regenerating Mouse Liver*

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Fundamental to the understanding and interpretation of histochemical and chemical changes in the liver following partial hepatectomy (18, 21) is a knowledge of the behavior of the cells during the process of restoration or regeneration. Some of the changes in the liver of various species including rats, following extirpation of two-thirds of the liver, have been described (3, 7, 9, 15, 17). The present study concerns the cytological changes in the livers of mice subjected to partial hepatectomy. Details concerning the mice and the method of operation have been given in the preceding paper (22).

MATERIALS AND METHODS

For the studies to be described in this paper, pieces of the right lateral lobe were fixed in Zenker-acetic fluid. After routine dehydration and imbedding in 56°–58° tissue mat, tissues were sectioned at 4 μ. Slides of each tissue were stained with hematoxylin and eosin, Feulgen reaction (14), and Heidenhain's azocarmine connective tissue stain.

Chalkley ratio methods (5) were employed for quantitative study of the liver tissue. Categories studied were liver cell nuclei, liver cell cytoplasm, and other elements (which included vascular space and all cellular and extracellular material other than liver parenchymal cells). From these data it was possible to determine what percentage of the volume of an idealized average liver cell was comprised of nuclear and cytoplasmic material. In addition, the proportion of liver tissue that was not actual parenchymal cells was obtained. Measurements were carried out by two observers, and the final data represent the averages of individual results on over 1,000 hits.

Nuclear and nucleolar diameters were measured with a filar ocular micrometer and the respective volumes calculated. Feulgen-stained sections were examined with light filtered with a Wratten No. 58 green filter to give sharper definition of the nuclear membrane and nucleolar-associated chromatin. The diameters of the nuclei were measured by moving the filar micrometer thread across the nucleus, including the nuclear membrane at both sides. The nucleoli were measured by placing the micrometer thread on the inner surface of the nucleolar-associated chromatin; it was desirable to measure only the nucleolus itself, excluding any chromatin. Volumes were calculated by assuming that the structures measured were spherical. The nucleolar volumes can be expressed in two ways: (*a*) average nucleolar volume, based on individual nucleoli; (*b*) average total nucleolar volume per nucleus, based on the sum of the volumes of the nucleoli in a single nucleus. Since there was some variation in the number of nucleoli per nucleus, it was felt that the second method of expressing nucleolar volume was preferable. Nuclear measurements were made by two observers; the final mean values were based on 100–200 nuclei. Nucleolar measurements were done by one observer; the mean volumes were based on measurements of all the nucleoli in at least 50 nuclei, with a minimum of 100 nucleoli being measured.

The incidence of mitotic activity, the percentage of liver cells that were binucleate, and the percentage of nuclei in the liver that were not parenchymal nuclei were counted. All the cells in the microscopic field were counted with an oil immersion objective and classified as to type, according to the major categories of parenchymal and nonparenchymal cell. Parenchymal cells were further classified as to whether or not they were binucleate, or dividing by mitosis. This was done for numerous fields, with random sampling of one or more sections, until a total of over 1,000 cells were counted. At least 500–600 liver cells were included in counts for each tissue. The data on nonparenchymal cells are presented in another paper (22).

OBSERVATIONS AND DISCUSSION

Mitotic activity.—The occurrence of mitotic cell division is one of the chief indices of liver restoration in terms of cell reproduction. Chart 1 summarizes the data on mitotic activity. The increase in mitotic activity begins at a variable rate in different animals. As early as 24 hours after partial hepatectomy one animal showed a low rate of mitotic activity. Even on the second day, however, two out of five tissues contained no mitotic figures. By the third day regeneration had started in all animals, and, in addition, the highest values were found at this time. Mitotic figures continued to be found in all animals, with a tendency to decrease in frequency, until the seventh day. The mitotic activity of regenerating liver measured at subsequent intervals of 3, 4, and 6 weeks and 2, 4, and 6 months was from 0 to 2 per cent and within the range of the livers at 14 days' regeneration. Apparently, in most cases the acute response of regeneration had terminated by the seventh day; four out of eight animals showed no mitotic activity, and in

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three other animals less than 1 per cent of the liver cells were in mitosis. Only one 7-day animal had an appreciable number of mitotic cells—over 4 per cent. It is of interest that the inclusion of this exceptional tissue in the group raises the mean mitotic activity to 0.7 per cent, as shown in Chart 1.

As described previously (22), this initial phase of recovery from partial hepatectomy is followed by a period of several weeks in which variable amounts of focal necrosis and associated leukocytic infiltration are observed. Evidently, the focal

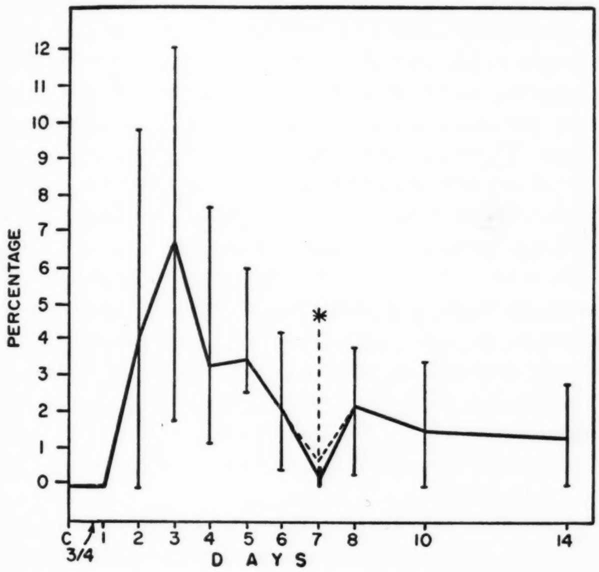

CHART 1.—Percentage of mouse liver cells in mitosis at intervals during regeneration. Dotted lines show change produced by one exceptional animal of eight in the 7-day group.

necrosis is severe enough to call forth secondary regenerative activity. It is felt that the exceptional 7-day animal just described, with the high mitotic activity, should fall within this group.

There is considerable fluctuation in mitotic activity through the fourth month. At all stages, however, there are some livers showing little or no mitotic activity. Presumably, the tissues with numerous mitotic figures are in the process of recovering from recent focal necrosis. The time of occurrence of the necrosis and its degree vary considerably from animal to animal. Therefore, while the curve for mitotic activity for the first 7 days may give a picture of the generalized response common to all animals, the subsequent activity through the fourth month should more properly be considered as showing the fluctuations and variations among the individual experimental animals. Finally, by the sixth month, the livers of various animals are again more uniform, and mitotic activity is essentially absent; in the livers of three animals no mitotic cells were observed, and one had 0.1 per cent mitotic cells.

No data were obtained for mitotic activity in cells other than liver parenchyma. Mitotic figures were observed, however, in bile duct cells, and in other nonparenchymal cells of the liver.

It is possible to speculate as to some of the factors (10, 19) related to the initiation and cessation of mitotic division. Among the factors influencing the rate of liver regeneration are species, diet, age, blood flow through the liver, and degree of partial hepatectomy. As has been reported previously (22), in mice the liver weight to body weight ratio falls immediately after the operation, due to the removal of two-thirds of the liver. However the ratio rapidly returns to normal, in some cases as early as the fourth day after partial hepatectomy. The liver itself still weighs less than those of normal controls, but the body weight is also lower. It seems suggestive that it is also on the fourth day that the mitotic activity starts to fall off. Perhaps one of the factors initiating liver regeneration is associated with the reduction in this ratio, which would represent a decrease in the amount of functional liver tissue relative to the body it serves. Furthermore, a return to normal of this ratio may play a part in stopping the rapid regeneration process.

Binucleate liver cells apparently undertook mitotic division as readily as mononucleate cells. Table 1 gives data on individual liver tissues that

TABLE 1

RELATIVE FREQUENCY OF BINUCLEATE LIVER CELLS AND BINUCLEATE PROPHASES

REGENERATION STAGE (DAYS)	MOUSE	PER CENT BINUCLEATE CELLS: All liver cells	PER CENT BINUCLEATE CELLS: Prophases	NO. PROPHASES COUNTED
2	1	22	38	21
	2	25	26	54
	3	22	42	12
3	1	6	22	62
	2	8	35	19
	3	18	33	6
	4	3	15	20
	5	6	9	11

substantiate this observation. Although the number of prophase cells counted was not so large as desirable for statistical validity, for convenience in comparison the results are expressed in percentages. The proportion of binucleate cells in prophase is comparable to the proportion of all liver cells that are binucleate. In fact, in many cases it appears that there may be an even greater proportion of binucleate cells undergoing mitotic division. Binucleate cell division will be discussed

further with other problems concerning the binucleate cells.

Marshak and Byron (11) found that the percentage of cells in mitosis increased from 0.03 to 0.37, 0.47, and 0.27 on the first, second, and third day of rat liver regeneration. Brues and Marble (4) found an increase from less than 0.001 per cent to 2.1 per cent mitotic cells at 24 hours with a decline towards normal during the next 2 days. Stowell (15) found the peak of mitotic activity at 30 hours of regeneration. Abercrombie and Harkness (1, 8) reported that the distribution of mitosis is similar in various lobes of the liver, although it may vary from one part of the liver lobule to another, and noted the incidence of mitoses in different types of cells in the rat liver at various stages of rat liver regeneration. They found that the greatest mitotic activity was represented by 2.87 per cent of the parenchymal cells at 1 day, and by 0.93 per cent of the littoral cells and 0.59 per cent of the bile duct epithelial cells on the second day of regeneration. The variations in results of different authors may be caused by the intervals at which counts were done, stages of mitosis included, as well as all the factors affecting the rate of regeneration, including age, species, diet, percentage of liver removed and general health of the animals. The regenerating mouse liver in these experiments showed a maximal mitotic activity on the third day (Chart 1).

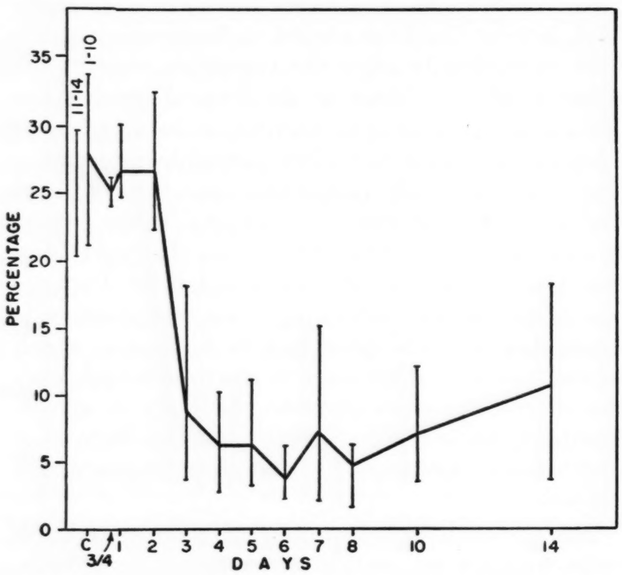

Chart 2.—Percentage of binucleate liver cells at intervals during regeneration.

Binucleate liver cells.—The data on frequency of binucleate cells represent the proportion of liver cells in 4-μ sections observed to contain two nuclei. These figures are low, for there was no correction to account for the error arising from not observing the nuclei in cells present in the plane vertical to the two dimensions principally seen in the 4-μ section. Although such errors (12, 13) would vary if there were substantial differences in the mean nuclear percentage of cells of different liver specimens, the data on binucleate liver cell counts are considered satisfactory for this comparison.

The proportion of liver cells that contain two nuclei in the early stages of regeneration is shown in Chart 2. Cells were observed containing three or more nuclei, but they were too infrequent to give reliable statistics and therefore were not included in the data. The two vertical lines near the left margin in Chart 2 show separately the distribution of counts in a group of ten and a second group of four control animals, which fall within the same range. There is a striking drop in the relative number of binucleate liver cells on the third day. The lowest range is found on the sixth day. There is an evident tendency toward restoration of normal values as early as the seventh day. The period of focal necrosis and infiltration already mentioned appears to interrupt the trend. However, occasional animals at regenerative stages of 1 and 2 months fall near the normal range, and almost all animals at the 3-, 4-, and 6-week and 2-, 4-, and 6-month stages are definitely over the range of the 6-day animals.

Because of its timing, it seemed highly probable that this decrease in number of binucleate cells is related to mitotic activity. As has already been shown (Table 1) the binucleate cells divide readily.

TABLE 2

Production of Binucleate Cells by Mitosis

Regeneration stage (days)	Mouse	Per cent binucleate cells: All liver cells	Per cent binucleate cells: Telophases	No. telophases counted
2	1	22	0	9
	2	22	0	3
3	1	6	12	74
	2	8	12	33
	3	18	63	11
	4	9	14	7
	5	6	25	16
	6	3	0	35

However, apparently a large proportion of these binucleate mitotic divisions produce polyploid mononucleate cells, for the proportion of binucleate telophases is low in most tissues. Table 2 gives data from individual animals that demonstrate this relationship. As in Table 1, the data on the percentage of telophases that are binucleate are also unreliable because of the small sample size; but the data reveal some idea of the order of

magnitude. It can be seen that, with one exception, the proportion of telophases that are binucleate is not high enough to maintain the control value of 28 per cent binucleate liver cells. In some cases the proportion of binucleate telophases is not even great enough to maintain the lower proportion of binucleate cells found in these tissues. There is one exceptional animal in the 3-day group which has an unusually high percentage of binucleate telophases. Since, however, it also has a much higher proportion of binucleate liver cells than in other livers at this stage, the exceptional animal substantiates the theory of the relationship between mitotic activity and reduction in numbers of binucleate cells. For some reason, the cells in this tissue were less able to carry through complete typical mitotic divisions; nuclear division is complete, but cytoplasmic division is not achieved. In general, it was found that, during the period of mitotic activity, tissues with unusually high numbers of binucleate cells also contained numerous binucleate telophases.

It is difficult to be certain of the explanation for the apparent increase in relative number of binucleate cells that occurs after the period of major mitotic activity is passed. The following observations, however, are relevant. During the period of greatest mitotic activity, and shortly thereafter, numerous bizarre, constricted nuclei are seen. They are undoubtedly the result of abortive, atypical mitotic divisions. They are found much less frequently in later recovered stages. It seems possible that the constrictions of such nuclei may break through and the resultant nuclear parts may undergo subsequent rounding; surface tension alone could account for this. Such regulation would result in cells with two or, less often, more nuclei. Furthermore, it is possible that as the stimulus to mitotic division decreases the number of cells able to carry through division to a successful completion may decrease.

St. Aubin and Bucher (13) found that the percentage of binucleate cells in regenerating rat liver dropped from 25 to 11 per cent by the third day and remained in the range of 8–11 per cent until the fourteenth day. Sulkin (17), using a technic similar to that employed in the present study, found 11.7 per cent binucleate cells in control rats and 4.95 per cent after 28 days' regeneration. The present data cannot be compared directly to those of the previous studies because of the different species studied. However, it is evident that there is a striking and relatively greater decrease in the proportion of binucleate cells in the mouse as compared to that in the rat. The data demonstrate the relationship of this decrease to mitotic activity of binucleate liver cells as well as the tendency to return toward normal values subsequent to the period of rapid proliferation.

Chalkley ratio data.—The results of Chalkley ratio measurements are given in Table 3. Only the percentage of the liver cell that is nuclear material is included; the value for cytoplasm is simply the difference between the relative nuclear volume and 100 per cent.

The changes in quantitative relationships in the liver parenchymal cell are fairly clear-cut during

TABLE 3

CHALKLEY RATIO MEASUREMENTS ON REGENERATING MOUSE LIVER

REGENERATION STAGE	PER CENT OF LIVER CELL THAT IS NUCLEAR MATERIAL		PER CENT OF LIVER THAT IS NOT PARENCHYMA	
	Mean	Range	Mean	Range
Control	7.7	7.0– 8.9	12.5	11.5–13.1
18 hours	8.5	7.7– 8.9	15.1	13.4–17.0
1 day	7.3	6.4– 7.7	14.0	13.0–15.2
2 "	8.3	7.2– 8.7	14.7	10.3–18.6
3 "	8.8	8.0–10.5	18.0	14.1–23.2
4 "	9.9	9.0–10.7	19.4	17.7–21.1
5 "	9.8	9.0–10.3	20.9	19.8–22.0
6 "	8.8	8.2– 9.4	20.9	18.5–23.6
7 "	8.6	7.5– 9.6	18.5	14.7–22.0
8 "	8.4	6.9–10.7	23.3	15.5–31.9
10 "	8.4	6.3– 9.5	19.6	16.0–23.9
14 "	8.1	6.6–10.2	17.6	12.1–28.0
21 "	8.7	7.7– 9.3	16.4	14.4–18.0
28 "	8.5	7.3– 9.8	19.5	14.6–27.1
6 weeks	8.0	7.2– 8.8	15.2	12.3–20.2
2 months	8.9	8.1–10.0	17.1	13.6–21.6
4 "	8.2	7.6– 9.3	15.6	13.6–18.6
6 "	7.8	7.4– 8.0	14.3	12.0–16.6

the period of rapid restoration, through the seventh day. For the first 3 days the proportion of the cell that is nuclear material is essentially unchanged; all tissues, with one exception at 1 day, fall within the control range, although the mean does vary. Beginning the third day, the percentage of the liver cell that is nuclear material increases, and it is in all cases above the control range on the fourth and fifth days. On the sixth and seventh days there is a return toward normal. It is evident that the values eventually fall within the normal range, except in a few individuals. These data, which are relative, will be discussed later with those concerning absolute volume changes of liver nuclei and cytoplasm.

There is an immediate rise in the proportion of the liver tissue that is not liver parenchymal cells. The initial rise in such elements may represent primarily vascular dilation and increased extracellular fluid. After the seventh day, high values may be accounted for by the areas of focal necrosis (which were classed as other elements rather than as liver cells) and the associated inflammatory cells and fibrosis. Apparently, even the recovered

livers do not return to control values. Since, however, as has been previously shown (22), the proportion of nonparenchymal cells does return to normal, the slightly higher amounts of nonparenchymal material in restored livers may be from noncellular connective tissue elements.

Changes in liver cell volume.—The nuclear and nucleolar volumes were measured directly, as already described. The mean cytoplasmic volumes are derived data; since the mean nuclear volume is known, and also the proportion of the cell that is nuclear and cytoplasmic material has been determined by the Chalkley ratio method (5), it is possible to calculate the volume of cytoplasm. The average cell volume can be determined by adding the nuclear and cytoplasmic volumes. There are several sources of error in this calculation, which are discussed in other papers (15, 16, 20); it is felt that they are reasonably constant and that, therefore, the data can be used for comparative purposes. The results are calculated in cubic microns for the fixed tissues, and the relationship of the fixed to living cell volumes is presumed to be reasonably proportional. The data are shown in Table 4.

TABLE 4

VOLUME CHANGES IN LIVER CELL CONSTITUENTS IN CUBIC MICRONS AT INTERVALS DURING REGENERATION

REGENERATION STAGE	NUCLEAR VOLUME Mean	Range	CYTOPLASMIC VOLUME Mean	Range	TOTAL NUCLEOLAR VOLUME PER NUCLEUS Mean	Range
Control	297	214–357	3,592	2,716–4,492	0.92	0.8–1.1
18 hours	430	352–537	4,745	3,644–6,433	1.60	1.4–1.8
1 day	289	202–347	3,641	2,958–4,163	1.10	1.0–1.3
2 days	374	219–577	4,132	2,452–6,053	1.06	0.6–1.4
3 "	402	353–446	4,266	3,804–4,683	1.08	0.7–1.6
4 "	435	385–474	3,991	3,355–4,481	1.15	0.9–1.6
5 "	497	444–571	4,573	3,909–5,258	1.93	1.4–3.1
6 "	459	396–516	4,727	3,860–5,554	1.27	0.8–1.5
7 "	456	378–584	4,554	4,041–5,328	1.21	0.8–1.9
8 "	486	388–606	5,192	4,522–6,541	1.35	1.1–1.9
10 "	427	342–471	4,746	4,276–5,088	1.66	1.0–2.6
14 "	552	478–662	6,690	4,302–9,368	2.03	0.8–3.5
21 "	528	410–660	5,590	3,999–7,911	1.15	0.9–1.6
28 "	445	384–553	4,875	3,608–5,957	1.45	0.8–2.5
6 weeks	397	322–517	4,647	3,558–6,756	1.04	0.7–1.6
2 months	381	296–428	3,961	2,664–4,595	0.98	0.7–1.2
4 "	388	277–518	4,452	2,702–6,302	0.92	0.8–1.0

There is a striking initial increase in nuclear volume at 18 hours, with a return to control values at 1 day. This increase is also observed in nucleolar and cytoplasmic volume and probably reflects a generalized response such as fluid intake. The nuclear volume subsequently shows a consistent increase, reaching a maximum at 5 days. There is a tendency to decrease on the sixth and seventh days. In some of the tissues showing focal necrosis, extremely high mean nuclear volumes were observed, but it is evident that there is a trend toward normal values at later stages.

The meaning of these volume changes is not clear. At least some of the increased volume may be related to increased fluid content. Also, it is possible that early prophases were unintentionally measured, although an effort was made to measure only intermitotic nuclei. The extremely large mean nuclear volumes found in occasional tissues may be associated with abnormal states, although those tissues did not necessarily show the most necrosis or infiltration. The fact that apparently the mean volume does not return to the control values probably reflects an increase in incidence of polyploid nuclei. Although no special study was made of such nuclei, it is certain that they must have been produced by mitotic division of binucleate cells in the cases where two mononucleate daughter cells were produced.

The data on cytoplasmic volume and cell volume show essentially the same changes, since the cytoplasm makes up the major part of the cell volume; the influence of changes in nuclear volume on cell volume, therefore, are slight. Only the data concerning the cytoplasmic volume are recorded here. There is an initial transient increase, comparable to that just described for the nuclei. Although the mean values increase slightly through the fourth day, only two of the animals are outside of the control range. The amount of cytoplasm is definitely increased, however, from the fifth day through the eighth day. Again, there is great variation among the animals after the eighth day. At later stages there is a return toward normal.

As in the case of the nuclear volume changes,

interpretation is not easy. It is possible that the slight increase in cytoplasmic volume through the fourth day (the period of maximum cell division) represents fluid uptake. In fact, since the nuclear percentage increases it is possible that there is actually less functional cytoplasm present during this period. This would be expected, since in the case of rapidly dividing cells the synthesis of new cytoplasm frequently does not keep pace with the nuclear reduplication. There is a definite increase in cytoplasmic volume in the tissues from 5 to 7 days, after cell division is slowing up; this substantiates the above interpretations. The extreme values in later stages appear to be abnormal; eventually there is a clear-cut trend toward control values.

The nucleolar volume also undergoes an initial increase. The subsequent decrease at 1 day is not quite back to normal, and there is little change through the fourth day. Since the nucleolar volume remains essentially unchanged during the period of maximum mitotic activity and is only slightly above normal, it seems probable that increased fluid content is one of the chief factors involved, rather than any more active functional process. The increase in mean volume at 5 days is striking. In the light of other investigations (15, 22) this increase in size may be associated with synthetic activities required for the production of increased amounts of functional cytoplasm already assumed to occur after the fourth day. There is a return towards normal through the seventh day. In later stages all tissues appear to have normal amounts of nucleolar material.

Ferreira (6) and Abercrombie and Harkness (1) found the nuclear volumes largest (170 per cent) at the second day of regeneration in rat liver, while Stowell (15), using younger animals, found the greatest volume at 24 hours (217 per cent) as compared to controls. In the mouse there was no single peak of nuclear volume increase, although the mean volumes were 145, 186, and 130 per cent of control volumes at $\frac{3}{4}$ day, 14 days, and 4 months, respectively. In the regenerating rat liver, Stowell (15) found that the mean nucleolar volume per nucleus had increased nearly 4 times by 24 hours, while the comparable data for the mice showed a mean volume of 174 per cent at $\frac{3}{4}$ day and 221 per cent at 14 days' regeneration, again illustrating the greater variability of the data in the mouse. Increase in cytoplasmic volume in regenerating rat liver (15) went to 258 and 239 per cent at 18 and 24 hours, as compared to 132 per cent at 18 hours and 187 per cent at 14 days in the mouse. The increase in hepatic parenchymal cell constituents is more erratic and less dramatic in the mouse than in the rat.

SUMMARY AND CONCLUSIONS

Cytological studies of the liver of the mouse following partial hepatectomy suggest that the recovery period should be divided into two phases. First, there is a stage of rapid restoration of liver cells, essentially complete by the seventh day. Then there follows a period of about a month in which occur varying amounts of secondary regenerative activity, in many instances associated with focal necrosis. There is an impressively successful recovery in almost all instances. Apparently the focal necrosis delays but does not prevent this final recovery.

Mitotic activity starts by the second day of regeneration and reaches a maximum on the third day. By the seventh day rapid cell multiplication is essentially completed. Mitotic activity is renewed sporadically when focal necrosis is present and replaces the cells lost. In the fully recovered livers no measurable mitotic activity is found.

The number of binucleate liver cells decreases abruptly on the third day. This is demonstrated to be related to mitotic activity as follows: (*a*) binucleate cells divide as readily as do the mononucleate cells; but (*b*) relatively few of the mitotic divisions produce binucleate cells. Therefore, binucleate cells are lost, by mitotic division, at a greater rate than they are formed. There is an increase in binucleate cells following the cessation of mitotic activity. Possible reasons for this are discussed.

The liver parenchymal cells are larger than normal during the period of greatest mitotic activity. This increase in size may be related to an increased water content of the cells, for the volume of both nuclei and cytoplasm is greater. From the fifth to seventh days, when the nuclear volume decreases, the cytoplasmic volume is even greater. Since the nucleolar volume increases strikingly on the fifth day, it is suggested that this is the time of major synthesis of new hepatic parenchymal cell cytoplasm. It is also at this time that the nucleo-cytoplasmic ratio begins to return to normal.

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On the Enhancement of the Carcinogenicity of 4-Dimethylaminoazobenzene by Fluoro-Substitution*

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The small size of the fluorine atom and the strong bond it forms with carbon have led us to use this element in assessing the importance of the ring positions of 4-dimethylaminoazobenzene (DAB) (Chart 1) to its carcinogenic activity towards the rat liver. In our initial study (17), the substitution of a single fluorine atom in either the 2′, 3′, or 4′ positions produced dyes which are more active than the parent dye. These results are in strong

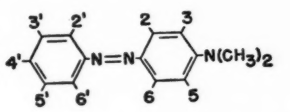

Chart 1.—The ring-positions of 4-dimethylaminoazobenzene (DAB).

contrast to those obtained on substitution of other groups which are larger or more easily metabolized (12, 13). Furthermore, these data suggested that through the use of appropriate fluoro derivatives of this dye it might be possible to determine whether or not certain ring positions must remain unsubstituted for carcinogenesis to occur.

We now wish to report on the carcinogenicities of several polyfluoro derivatives of DAB involving the equivalent positions of the prime ring. As with the monofluoro derivatives, all these compounds are more active carcinogens than the unsubstituted dye. We interpret this to mean that no position on the prime ring of this dye is directly concerned in the carcinogenic process. These results also suggest that the effects of systematic fluoro-substitution on the potency of other biologically active molecules could furnish strong evidence as to which positions do or do not contribute directly to the activity in question.

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METHODS

Preparation of compounds.[1]—All the fluoro amines and dyes employed in these experiments were prepared in these laboratories. The 2′-, 3′-, and 4′-fluoro derivatives of DAB have already been described (17). The other dyes are new, and analytical data for these compounds are given in Table 1. All the

TABLE 1

Analyses on New Fluoro Derivatives of DAB

-DAB	M.P. (uncorr.)	Per cent N (Dumas)* Found	Calculated
2-Fluoro-	107.5°–108°	17.24, 17.29	17.27
2′,4′-Difluoro-	132°–133°	15.75, 15.78	16.09
2′,5′- "	135.5°–136°	16.02, 16.11	"
3′,5′- "	110.5°–111°	15.98, 16.02	"
2′,4′,6′-Trifluoro-	111°–112°	14.76, 14.80	15.05

* These determinations were made by the Clark Microanalytical Laboratory, 104½ West Main Street, Urbana, Ill.

dyes were purified by passing 10–20-gm. portions in benzene-petroleum ether solution through alumina[2] columns 3 cm. in diameter and 40 cm. in length. Approximately 50 mg. of each dye was carefully chromatographed on an alumina column under similar conditions prior to analysis for nitrogen.

2-Fluoro-DAB.—The methylation procedure of Hodgson and Nicholson (7) was employed to convert 3-fluoroaniline (17, 24) to the tertiary amine. The latter amine was then coupled with diazotized aniline (17). A yield of 50 per cent was obtained.

2′,4′-, 2′,5′-, and 3′,5′-Difluoro-DAB.—These dyes were prepared by coupling the diazonium salts of the corresponding amines with dimethylaniline (17). 2,4-Difluoroaniline (23, 27) was diazotized as described previously (17). 2,5-Difluoroaniline (26) was diazotized according to the procedure of Saunders (22) for the corresponding chloro derivative. 3,5-Difluoroaniline was prepared and diazotized by the procedure of Finger *et al.* (5) All the dyes were obtained in yields of approximately 80 per cent.

2′,4′,6′-Trifluoro-DAB.—2,4,6-Trifluoroaniline was prepared according to Finger *et al.* (5). The diazotization of this amine and the coupling with dimethylaniline were performed as described for the corresponding chloro compound (17, 22).[3] A yield of 48 per cent was obtained.

[1] We are indebted to Miss Shirley Fraser and Mr. Ralph Sapp for aid in the preparation of some of these compounds.

[2] Merck, "Suitable for Chromatographic Adsorption."

[3] The quantity of anhydrous sodium acetate given in (17) for the preparation of 2′,4′,6′-trichloro-DAB is in error and should be 3 times as great, or 246 gm.

Assay procedure.—Young adult male albino rats[4] weighing 180–200 gm. were housed in screen-bottomed cages in groups of 5–7, with food and water available ad libitum. All the compounds were fed in a semisynthetic diet (11, cf. diet 3) consisting of: crude casein, 12; Vitab rice bran concentrate, 2; salts, 4; glucose monohydrate (cerelose), 77; and corn oil, 5. After analysis of the casein and Vitab sufficient crystalline riboflavin was added to bring the total content of the diet to 2 mg/kg. The dyes were dissolved in the corn oil with mild heat. Sodium fluoroacetate was added as a glucose triturate to the diets for groups 14 and 15 (Table 2).

Groups of sixteen rats each were fed the dyes for the times and at the levels given in Table 2. At the time intervals noted, the livers were examined for tumors and for the degree of cirrhosis by laparotomy. The animals were then fed the diet with no dye added for an additional 2 months. At this time they were killed for a final tumor count. In Series I–III all the dyes were fed at a level of 2.40 mM/kg of diet which is equivalent to 0.054 per cent DAB. In the cases of 2-fluoro-DAB and 2′,4′-difluoro-DAB (Series II), the initial weight losses were large, and it was necessary to remove the dyes from the diet for 1 week at the end of the first and the second months. In each case, however, the dye was fed for a total of 3 months. Similarly, in Series IV the trifluoro dye proved sufficiently toxic at an initial level of 0.066 per cent that it was necessary to feed the dye-free diet for 1 week after the first week of dye feeding and, thereafter, to feed the dye at a level of 0.049 per cent. In this case, the control rats receiving DAB were similarly treated.

TABLE 2

THE CARCINOGENICITIES OF VARIOUS FLUORO DERIVATIVES OF 4-DIMETHYLAMINOAZOBENZENE

Series	Group	Compound fed	Per cent in diet	Time compound was fed (mo.)	Incidence of liver tumors* (mo.) 3	4	5	6	Gross cirrhosis at end of feeding compound
I	1	DAB	0.054	3	2/15		7/15		none-mild
	2	2′-Fluoro-DAB	0.059	"	4/13		8/13		mild
	3	3′- " "	"	"	8/14		12/14		moderate
	4	4′- " "	"	"	16/25		24/25		"
II	5	DAB	0.054	3	2/16		5/16		none-mild
	6	2-Fluoro-DAB	0.059	"	3/15		13/15		mild-moderate
	7	2′,4′-Difluoro-DAB	0.063	"	10/16		16/16		moderate-severe
III	8	DAB	0.054	4		3/16		11/16	none-mild
	9	2′,5′-Difluoro-DAB	0.063	3	9/16		16/16		moderate
	10	3′,5′- " "	"	"	9/14		14/14		"
IV	11	DAB	0.054† −0.045	3	1/15		6/15		none-mild
	12	2′,4′,6′-Trifluoro-DAB	0.066† −0.049	"	5/15		13/15		moderate-severe
V	13	DAB	0.06	4		2/15		7/15	none-mild
	14	" + sodium fluoroacetate	0.06† 0.002	"		3/15		7/15	" "
	15	Sodium fluoroacetate	0.002	10				0/16 (and at 10 mos.)	none

* No. animals with tumors/number of animals alive at end of dye feeding.
† See assay procedure.

RESULTS

The carcinogenic activities of the various fluoro derivatives of DAB are given in Table 2. A control group fed an equimolar level of the unsubstituted dye was included in each series. The tumor incidences in these groups were reasonably consistent. The time of dye feeding was kept short, and the level of riboflavin in the diet was held at a moderately high level so that the tumor incidence in the control groups would not be too high and thus obscure the high activities expected for most of the fluoro dyes.

Series I is essentially a repetition of earlier experiments (17) and agrees well with the latter, even on the activity of 2′-fluoro-DAB, which again proved to be slightly more active than DAB. The high activity of the 4′-fluoro derivative has now been demonstrated several times (17, 21) and is of interest since only one position of this type (*para*) exists in the prime ring. The only new monofluoro dye tested in these experiments was 2-fluoro-DAB (group 6), which is also a very active carcinogen.

All three difluoro dyes, 2′,4′-, 2′,5′-, and 3′,5′-difluoro-DAB (Series II and III) proved to be strong carcinogens. The 3′,5′-derivative is of special interest, since both equivalent *meta* prime positions are substituted in this molecule.

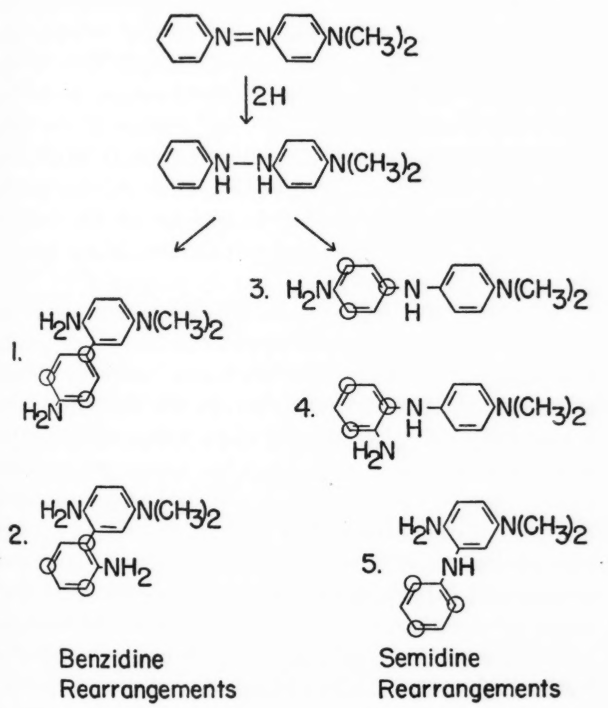
Even the most highly substituted dye, 2′,4′,6′-trifluoro-DAB (Series IV), exhibited a higher degree of activity than the parent dye. It should be noted that both equivalent *ortho* prime positions are substituted in this derivative. In addition, blocking the three *ortho* prime and *para* prime positions should prevent four of the five possible rearrangements which the hydrazo derivative of DAB could undergo *in vivo* (Chart 2).

Series V was designed to test the possibility that

[4] Holtzman-Rolfsmeyer Rat Company, Madison, Wis.

a portion of these fluoro dyes might be metabolized to fluoroacetate (cf. 9). This compound is metabolized to form a specific enzyme inhibitor (2, 20), and, hence, if formed, might have a cocarcinogenic action which would account for the general high activity of the fluoro dyes. Unfortunately, the maximum level of sodium fluoroacetate which our rats tolerated for extended periods was only 20 mg/kg of diet; this is only about one-thirteenth of the maximum amount of fluoroacetate that could be formed from a monofluoro-DAB fed at a level of 0.06 per cent. In any event, no effect of the added fluoroacetate on the toxicity and carcinogenicity of DAB was observed. Furthermore, it appears unlikely that the fluoro dyes are metabolized to significant amounts of fluoroacetate, since none of the fluoro dyes appeared to be any more toxic than other highly active carcinogenic dyes such as 3′-methyl-DAB (12).

Table 3 contains the *approximate* activities of



O = positions blocked in 2′,4′,6′-trifluoro-DAB

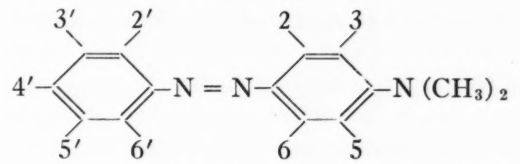
CHART 2.—The possible rearrangement products of 4-dimethylaminohydrazobenzene.

the fluoro derivatives estimated as described previously (13) and compares these with previous results for other substituents (12, 13, 17).

As noted before for many derivatives of DAB, the extent of gross cirrhosis in the livers of the rats fed these fluoro derivatives paralleled the observed carcinogenic activities. All the tumors continued to grow after the period of dye-feeding and eventually caused the death of the animals. Histologically, these tumors were similar to those produced by DAB.[5] The detailed pathology in the livers of rats fed 4′-fluoro-DAB has been presented elsewhere by Price *et al.* (21).

TABLE 3

THE CARCINOGENICITIES OF VARIOUS RING-SUBSTITUTED DERIVATIVES OF 4-DIMETHYLAMINOAZOBENZENE*



POSITION	RELATIVE ACTIVITIES (UNSUBSTITUTED DYE = 6) F-	CH_3-	Cl-	Br-	NO_2-	CF_3-	HO-
4′	10–12	<1	1–2		0‡	0	0
3′	10–12	10–12	5–6	(0)†	5‡	0	0
2′	7	2–3	2		3	0	0
2	>10	0					0
3		0					
2′,4′	>10	0					
2′,5′	>10	0	0				
3′,5′	>10	0					
2′,4′,6′	>10		0	0‡			

* Data from Refs. 12, 13, 17, and present paper.
† Kuhn and Quadbeck (8).
‡ Poorly absorbed.

DISCUSSION

The very high carcinogenicities of 4′-fluoro-DAB, 3′,5′-difluoro-DAB, and 2′,4′,6′-trifluoro-DAB furnish strong positive evidence that neither the 4′ position nor either of the two pairs of equivalent positions, (3′,5′) and (2′,6′), can be directly involved in the carcinogenic process. Thus, if reactions essential to the carcinogenic process occurred *in vivo* at these positions, the substitution of fluorine for hydrogen should have prevented or at least hindered the formation of tumors. The high activities of the four remaining fluoro derivatives in which the prime ring is substituted add further weight to this conclusion. Furthermore, the possibility seems very remote that all or most of the prime positions could function equally well in a hypothetical carcinogenic reaction involving this ring. A test of this possibility would involve the preparation of prime-pentafluoro-DAB, although it is quite likely that excessive substitution with fluorine would decrease the over-all reactivity of the molecule and give a negative result of little value.

The conclusion that the prime ring of DAB is not directly involved in the carcinogenic process is in harmony with our studies (10) on the protein-bound dye which is formed from DAB *in vivo*. The

[5] We are indebted to Drs. H. P. Rusch and J. M. Price for the histological examination of these tumors.

prime ring is unaltered in the protein-bound dye which, on the basis of several correlations, appears to be of causal significance in the carcinogenic process induced in the liver.

The great enhancement of activity noted with seven of the eight fluorinated dyes is likely the result of at least two factors: (*a*) the blocking of reactions which normally inactivate a portion of the dye fed and (*b*) the promotion of carcinogenic reactions involving other sites in the molecule, e.g., the N-methyl groups (10, 14). For example, the high activity of the 4′-fluoro derivative would appear to involve chiefly the first factor, since hydroxylation to the noncarcinogenic 4′-hydroxy dye (13, 25) is known to occur in the rat liver (18). It is of interest that the activity of the extremely weak carcinogen, 4-aminoazobenzene, is enhanced by substitution of the 4′ position with fluorine. In this case, the increased activity may be related to the greater ability of the fluoro dye to become methylated on the amino group *in vivo* and subsequently form protein-bound dye (15).

A previous publication (17) presented the evidence against the possibility that a benzidine rearrangement (3, 4) of the hydrazo derivative of DAB was involved in the carcinogenic process. The strong carcinogenic activity of 2′,4′,6′-trifluoro-DAB now appears to rule out definitely the participation of both possible benzidine rearrangements and two of the three possible semidine rearrangements (Chart 2). The remaining semidine rearrangement (No. 5, Chart 2) may not be of importance in view of the high activity of 2-fluoro-DAB. However, the carcinogenic activity of 2,6-difluoro-DAB, when obtained, should provide a more cogent test of this point.

The relatively unique effect of fluorine substitution in the DAB molecule is seen in Table 3, which summarizes the results obtained with derivatives (12, 13, 17) that have been tested under comparable conditions. In particular, note the contrast of fluorine with methyl group substitution which yields a wide range of activities and a high proportion of inactive compounds. Similarly, the contrast of fluorine with the other halogens is great. Undoubtedly a large number of these differences are a result of the small size of the fluorine atom (19). On general grounds, interference with a carcinogenic reaction could be produced by the larger and more complicated groups in any or all of the following ways: (*a*) a purely steric effect involving a "poor fit" of the whole molecule, (*b*) direct blocking of the reaction at the substituted site, (*c*) depression of the reactivity of distant vital sites through electronic effects, and (*d*) metabolism of the group *in situ* (e.g., $-CH_3$ to -COOH, $-NO_2$ to $-NH_2$), followed by interference as in *a* to *c*.

It is unlikely that the fluorine atoms are removed *in vivo* from these dyes. The fluorine-carbon bond is among the strongest known (19), and in aromatic rings this bond is known to become labile only when strongly negative groups (e.g., $-NO_2$) are present in the *o*- and *p*-positions. However, even on the assumption that splitting of this bond does occur *in vivo* to a greater or lesser extent, it is most unlikely that these eight fluorinated dyes would have such high carcinogenic activities as a result of defluorination at so many different positions.[6] The inability of fluoroacetate at the level tested to affect the rate of tumor production by DAB argues against the possibility that this substance could be a cocarcinogenic metabolite of these dyes.

While it appears that none of the prime positions in the DAB molecule are directly involved in the carcinogenic process, little is known about the positions on the other ring. Only one fluoro derivative, 2-fluoro-DAB, has been tested which involves any of these positions, although it is of interest that in this case high carcinogenic activity was observed again. In any case, either of the two pairs of equivalent positions, (2,6) and (3,5), on this ring could be involved in the carcinogenic process. Attempts at the synthesis of the corresponding difluoro dyes and of the remaining monofluoro dye, 3-fluoro-DAB, are in progress.

The ability of fluoro substitution to enhance the activity of DAB does not appear to be restricted to carcinogens of this type. We have recently noted in collaborative work with Dr. R. B. Sandin of the University of Alberta (16) that 7-fluoro-2-acetylaminofluorene is a considerably stronger hepatocarcinogen in the rat than is the parent compound. Again it is of interest that hydroxylation occurs *in vivo* at this para position (1) to yield an inactive compound (6). Furthermore, it seems possible that more information on the importance of the various positions in the carcinogenic polycyclic aromatic hydrocarbons could be obtained with fluoro derivatives than has been possible with the many methyl derivatives that have been investigated.

Probably much useful information could be gained by testing the fluoro-derivatives of other biologically active molecules. If a fluoro-substituted molecule retained considerable activity, then the position(s) involved would not appear to be directly concerned in the mechanism which pro-

[6] In a subsequent experiment it has been found that the addition of 0.034 per cent of sodium fluoride to a diet containing 0.054 per cent of DAB did not alter the tumor incidence. This level of fluoride is the amount that could theoretically be liberated from an equimolar amount of a difluoro-DAB.

duced the observed activity. On the other hand, if a fluoro-substitution greatly diminished or destroyed the activity, then the position in question either might be directly involved or might be a position where electronic and possibly steric effects of the fluorine atom diminish the effectiveness of groups elsewhere in the molecule. This reasoning probably applies best to monofluoro derivatives or to poly-substituted molecules where only enough fluorine atoms are used to block equivalent positions. As far as we are aware, the use of fluorine substitution as an indicator of biologically active or inactive positions is new.

SUMMARY

In an investigation on the importance of the ring positions of 4-dimethylaminoazobenzene to its carcinogenic activity, the following new compounds were synthesized and tested in the rat: 2-fluoro-, 2′,4′-difluoro-, 2′,5′-difluoro-, 3′,5′-difluoro-, and 2′,4′,6′-trifluoro-4-dimethylaminoazobenzene. All these compounds were found to be more active liver carcinogens than the parent dye. The same finding had been made previously for the 2′-, 3′-, and 4′-fluoro derivatives. On the basis of these results it appears that no position on the prime ring of 4-dimethylaminoazobenzene is directly concerned in the carcinogenic process. The other ring remains to be investigated by this method.

The results further suggest that the effects of systematic fluoro-substitution on the activities of other biologically active molecules could furnish strong evidence on which positions do or do not contribute directly to the activity in question.

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Book Reviews

Die Frühdiagnose des Uteruscarcinoms: Histologie, Kolposkopie, Cytologie, biochemische Methoden. 2d rev. ed. By PROF. DR. H. LIMBURG. Stuttgart, Germany: Georg Thieme Verlag, 1952. Pp. 208. DM 19.50.

Within 1 year after the first edition, a second one has proved necessary. It is felt that nothing could better demonstrate the value as well as the necessity for this monograph. Despite all the effort expended to find some kind of cure for cancer, nothing has proved to be of definite value. Under these circumstances, it is only natural for clinicians to concentrate on the means of possible prevention and earliest diagnosis. This book, therefore, should be extremely useful for the general practitioner, as well as for the gynecologist, in his effort to diagnose carcinoma of the uterus at a time when surgery and radiation therapy are still able to achieve good results.

The major part of this book is concerned with early diagnosis from the morphological point of view. From the author's experience, it seems to be a great improvement to combine the cytodiagnosis (Papanicolaou) with colposcopy, including biopsy. The morphological part is accompanied by many excellent photographic reproductions of histological specimens.

The concluding part of this monograph, dealing with biochemical methods, leaves considerable room for discussion. The diagnostic values of measuring anaerobic glycolysis of tumor-suspicious tissue still remains somewhat uncertain. The applicability of the latter method is confined to hospitals with special equipment and trained personnel for it.

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Das Phaeochromozytom. By PROF. DR. H. SACK. Stuttgart, Germany: Georg Thieme Verlag, 1951. Pp. 93. Deutschemark 11.70.

The author presents a well written monograph on the pathology and the clinic of the Pheochromocytoma. This book seems to be very helpful in the understanding of this disease, since the material presented is considered from the point of view of pharmacology as well as of physiological chemistry. This monograph should be of special interest for the clinical pathologist.

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Ultraviolet Radiation. By LEWIS R. KOLLER. New York: John Wiley & Sons, Inc., 1952. Pp. 270. $6.50.

INFORMATION FOR AUTHORS

Manuscripts for *Cancer Research* must represent new and original contributions, not previously published, and, if accepted, not to be published elsewhere. They should be typed, double-spaced, with ample margins, on bond paper. The original and one carbon copy should be submitted. Manuscript pages should be numbered consecutively. Every paper should end with a brief, logical *summary* in which the points proved by the investigation are outlined.

Critical reviews on subjects dealing with cancer and allied fields will be considered for publication in *Cancer Research*. Such reviews should attempt to correlate the various approaches to a problem in a manner that will indicate the trends and emphasize the aspects that require further clarification. Critical analyses of the pertinent issues and expressions of opinions are encouraged. Preferably, the reviews should not exceed 7 pages (6,000 words) in length, including references.

Comments: A limited space will be available for the publication of comments on published data in the cancer field; they should not exceed 500 words. Comments written for the presentation of new data will not be published.

The title of the paper should be as short as is compatible with a clear indication of the subject matter. Chemical formulas should not be included in the title. The title of the paper, the name of the author (or authors), location, and any acknowledgments should appear on a separate page.

Footnotes should be numbered consecutively and should appear on a separate sheet.

Tables should be typed on separate sheets and should be numbered. Each must have a brief descriptive title. Tables should be arranged for vertical position on the page, either in single or double column. Every column must be supplied with an appropriate heading.

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Half-tone illustrations (photomicrographs and photographs) are designated *figures* and are printed as plates at the end of each article. Figures in moderate numbers will be accepted if they are of good technical quality and essential for the clarity of the presentation. Plates in color and excessive numbers of figures, if accepted, will be charged to the author. Only clear glossy photographs should be submitted. Mount all photographs tightly together on white cardboard. Place as many together as will make a plate not to exceed $6 \times 8\frac{1}{2}$ inches. Each figure should include the appropriate number on a lower corner, and they should be numbered consecutively starting with Figure 1. Legends for plate figures should be typed separately in consecutive order on standard-sized paper with the heading "Legends for Figures."

Line cut illustrations (graphs and charts) are designated *charts* and are printed in the text. These should be numbered in consecutive order starting with Chart 1. Charts should be planned so as to eliminate waste space; they should be ruled off close to the area of the curves, and descriptive matter should not extend beyond the curves. Charts should be so prepared that no letter or figure will be less than 2 mm. high after reduction to the size in which it will appear. Generally they will be reduced to fit either the one- (3-inch) or two- (6-inch) column width of the page. Original line drawings on high quality white drawing paper or board, blue tracing cloth, or coordinate paper *printed in blue* should be used. Typewritten charts are not desirable, as they permit but slight reduction. Only India ink should be used for drawing or lettering. One set is sufficient. Legends for each chart should be typed separately in consecutive order on standard-sized paper with the heading "Legends for Charts."

Mounts of charts and photographs larger than $8\frac{1}{2} \times 11$ inches will not be accepted. When original drawings exceed this size, photographed reductions should be sent.

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